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Signature: Kimberly W Zuehlke

Typed Name of Person Mailing Paper or Fee: Kimberly W. Zuehlke

UTILITY PATENT APPLICATION TRANSMITTAL

(Only for new nonprovisional applications under 37 CFR 1.53(b))

Attorney Docket No.	06510/060DIV	Total Pages	84
First Named Inventor or Application Identifier			
Shaun R. Couglin; Hiroaki Ishihara; Andrew Connolly			
Express Mail Label No.	EL 105 872 719 US		

APPLICATION ELEMENTS

See MPEP chapter 600 concerning utility patent application contents

1. X Fee Transmittal Form
   (Submit an original, and a duplicate for fee processing)
2. X Specification Total Pages 43
   (preferred arrangement set forth below)
   - Descriptive title of the invention
   - Cross Reference to Related Applications
   - Statement Regarding Fed sponsored R & D
   - Reference to Microfiche Appendix
   - Background of the Invention
   - Brief Summary of the Invention
   - Brief Description of the Drawings (if filed)
   - Detailed Description
   - Claim(s)
   - Abstract of the Disclosure
3. X Drawing(s) *(35 USC 113)* Total Sheets 16
4. X Oath or Declaration Total Sheets 2
   a. ___ Newly executed (original or copy)
   b. X Copy from a prior application (37 CFR 1.63(d) (for continuation/divisional with Box 17 completed)
   [Note Box 5 below]
   i. ___ DELETION OF INVENTOR(S)
   Signed statement attached deleting inventor(s) named in the prior application, see 37 CFR 1.63(d)(2) and 1.33(b)
   c. ___ Unsigned
5. X Incorporation By Reference *(useable if Box 4b is checked)*
   The entire disclosure of the prior application, from which a copy of the oath or declaration is supplied under Box 4b, is considered as being part of the disclosure of the accompanying application and is hereby incorporated by reference therein.

Address to: Assistant Commissioner for Patents
Box Patent Application
Washington, D.C. 20231

6. ___ Microfiche Computer Program (*Appendix*)
7. Nucleotide and/or Amino Acid Sequence Submission
   (*if applicable, all necessary*)
   a. ___ Computer Readable Copy
   b. ___ Paper Copy (identical to computer copy)
   c. ___ Statement verifying identity of above copies

ACCOMPANYING APPLICATION PARTS

8. ___ Assignment Papers (cover sheet & document(s))
9. ___ 37 CFR 3.73(b) Statement ___ Power of Attorney
   (when there is an assignee)
10. ___ English Translation Document *(if applicable)*
11. ___ Information Disclosure Statement (IDS)/PTO-1449 ___ Copies of IDS Citations
12. X Preliminary Amendment
13. X Return Receipt Postcard (MPEP 503)
    (Should be specifically itemized)
14. ___ Small Entity Statement(s) ___ Statement filed in prior application Status still proper and desired
15. ___ Certified Copy of Priority Document(s)
    (if foreign priority is claimed)
16. X Other: Submission of Formal Drawings; 16 Sheets of Formal Drawings

17a. If a CONTINUING APPLICATION, *check appropriate box and supply the requisite information:*
___ Continuation X Divisional ___ Continuation-in-part (CIP) of prior application No. 08/742,440

17b. If a CONVERSION from a PROVISIONAL APPLICATION, *supply the requisite information:*
Conversion of prior provisional application No. 60/_____, filed .

UTILITY PATENT APPLICATION TRANSMITTAL

(Only for new non-provisional applications under 37 CFR 1.53(b))

18. CORRESPONDENCE ADDRESS					
___ Customer Number or Bar code label	(Insert Customer No. or attach bar code label here)			or _X_ Correspondence address below	
NAME	**BOZICEVIC & REED LLP**				
ADDRESS	**285 Hamilton Avenue, Suite 200**				
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NAME	Dianna L. DeVore, Registration No. 42,484
SIGNATURE	Dianna L DeVore
DATE	12/8/98

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Signature: Kimberly W Zuehlke

Typed Name of Person Mailing Paper or Fee: Kimberly W. Zuehlke

FEE TRANSMITTAL

Note: Effective October 1, 1997.
Patent fees are subject to annual revision.

TOTAL AMOUNT OF PAYMENT ($)916.00

Complete if Known	
Application Number	**To Be Assigned**
Filing Date	**December 8, 1998**
First Named Inventor	**Shaun R. Coughlin et al.**
Group Art Unit	Not Assigned Yet
Examiner Name	Not Assigned Yet
Attorney Docket Number	06510/060DIV

METHOD OF PAYMENT (check one)

1. X The commissioner is hereby authorized to charge any **additional** fees and credit any overpayments to:
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Deposit Account Name: Bozicevic & Reed LLP

___ Charge Any Additional Fee Required Under 37 CFR 1.16 and 1.17

___ Charge the Issue Fee set in 37 CFR 1.18 at the mailing of the Notice of Allowance

2. X Payment Enclosed:
X Check ___ Money Order ___ Other

FEE CALCULATION

1. FILING FEE

Large Fee Code	Large Entity Fee ($)	Small Fee Code	Small Entity Fee ($)	Fee Description	Fee Due
101	760	201	380	Utility filing fee	$760.00
102	310	206	155	Design filing fee	
104	480	207	240	Plant filing fee	
109	760	208	380	Reissue filing fee	
110	150	214	75	Provisional filing fee	
				SUBTOTAL (1)	760.00

2. CLAIMS

	Extra		Fee from below	Fee Due
Total Claims 7 - 20 =	0	X	18	0.00
Independent Claims 5 - 3 =	2	X	78	156.00
Multiple Dependent Claims	0	X	260	0.00

Large Fee Code	Large Entity Fee ($)	Small Fee Code	Small Entity Fee ($)	Fee Description
103	18	203	9	Claims in excess of 20
102	78	202	39	Independent Claims in excess of 3
104	260	204	130	Multiple Dependent Claim
109	78	209	39	Reissue independent claims over original patent
110	18	210	9	Reissue claims in excess of and over original patent

SUBTOTAL (2) $156.00

FEE CALCULATION (continued)

3. ADDITIONAL FEES

Large Fee Code	Large Entity Fee ($)	Small Fee Code	Small Entity Fee ($)	Fee Description	Fee Due
105	130	205	65	Surcharge - late filing fee or oath	
127	50	227	25	Surcharge - late provisional filing fee or cover sheet	
139	130	139	130	Non-English specification	
147	2,520	147	2,520	For filing a request for reexamination	
112	920*	112	920*	Requesting publication of SIR prior to Examiner action	
113	1,840*	113	1,840*	Requesting publication of SIR after Examiner action	
115	110	215	55	Extension for reply within first month	
116	380	216	190	Extension for reply within second month	
117	870	217	435	Extension for reply within third month	
118	1,360	218	680	Extension for reply within fourth month	
128	1,850	228	925	Extension for reply within fifth month	
119	300	219	150	Notice of Appeal	
120	300	220	150	Filing a brief in support of an appeal	
121	260	221	130	Request for oral hearing	
138	1,510	138	1,510	Petition to institute a public use proceeding	
140	110	240	55	Petition to revive - unavoidable	
141	1,210	241	605	Petition to revive - unintentional	
142	1,210	242	605	Utility issue fee (or reissue)	
143	430	243	215	Design issue fee	
144	580	244	290	Plant issue fee	
122	130	122	130	Petitions to the Commissioner	
123	50	123	50	Petitions related to provisional applications	
126	240	126	240	Submissions of Information Disclosure Stmt	
581	40	581	40	Recording each patent assignment per property (times number of properties)	
146	760	246	380	Filing a submission after final rejection (37 CFR 1.129(a))	
149	760	249	380	For each additional invention to be examined (37 CFR 1.129(b))	
Other fee (specify)					
Other fee (specify)					

*Reduced by Basic Filing Fee Paid SUBTOTAL (3) ($)

SUBMITTED BY				Complete (if applicable)	
Typed or Printed Name	Dianna L. DeVore			Reg. Number	42,484
Signature	Dianna L. DeVore	Date	12/8/98	Deposit Account	18-0580

"Express Mail" Mailing Label No. EL 105 872 719 US
Date of Deposit December 8, 1998

Atty Docket No. 06510/060DIV

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Kimberly W. Zuehlke
Typed or Printed Name of Person Mailing Paper or Fee

Kimberly W Zuehlke
Signature of Person Mailing Paper or Fee

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of:

Shaun R. Coughlin et al.

Group Art Unit: Unassigned

Serial No.: Unassigned (Divisional of USSN 08/742,440)

Examiner: Unassigned

Filing Date: December 8, 1998

Title: PROTEASE-ACTIVATED RECEPTOR 3 AND USES THEREOF

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

This is a preliminary amendment to the patent application identified above. Prior to examination of the subject application, please enter the following amendments to the specification and claims:

IN THE SPECIFICATION:

On page 1, beneath the title, please insert the following:

--CROSS-REFERENCE

This application is a divisional application of Serial No. 08/742,440, filed October 30, 1996 which is incorporated herein by reference in its entirety and to which application we claim priority under 35 U.S.C. §120.--

IN THE CLAIMS:

Please cancel claims 1-4, 9-12 and 16-20 without prejudice.

REMARKS

Claims 5-8 and 13-15, which correspond to Restriction Groups II and IV, respectively, from the prosecution of parent application 08/742,440, are now pending in this application.

Original claims 1-4, 9-12 and 16-20 have been canceled from the application

During the prosecution of the parent application a restriction requirement was issued which resulted in the cancellation of original claims 5-8 and 13-15. This application has been filed to claim subject matter encompassed by these canceled claims.

In the event any fees are due in connection with the filing of this preliminary amendment or attached application or if petitions are required, applicants petition for any required relief and authorize the Commissioner to charge the cost of such petitions or other fees to our Deposit Account No. 18-0580.

Respectfully submitted,

Date: 12/8/98

By: Dianna L. DeVore

Dianna L. DeVore
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F:\DOCUMENT\6510 - UCAL\060DIV\Preliminary Amendment.wpd (Rev. 6/19/97)

APPLICATION

FOR

UNITED STATES LETTERS PATENT

TITLE: **PROTEASE-ACTIVATED RECEPTOR 3 AND USES THEREOF**

APPLICANT: **SHAUN R. COUGHLIN**
HIROAKI ISHIHARA
ANDREW CONNOLLY

PATENT
ATTORNEY DOCKET NO: 06510-060001

PROTEASE-ACTIVATED RECEPTOR 3 AND USES THEREOF

Field of the Invention

This invention relates to nucleic acids, their encoded protease-activated receptor 3 proteins, and screening assays for agonists and antagonists of the protease activated receptor 3 proteins.

Background of the Invention

Thrombin, a coagulation protease generated at sites of vascular injury, activates platelets, leukocytes, and mesenchymal cells (Vu, T.-K.H. et al. (1991) Cell 64:1057-1068). Activation of platelets by thrombin is thought to be critical for hemostasis and thrombosis. In animal models, thrombin inhibitors block platelet-dependent thrombosis, which is the cause of most heart attacks and strokes in humans. Available data in humans suggests that thrombosis in arteries can be blocked by inhibitors of platelet function and by thrombin inhibitors. Thus it is likely that thrombin's actions on platelets contribute to the formation of clots that cause heart attack and stroke. Thrombin's other actions on vascular endothelial cells and smooth muscle cells, leukocytes, and fibroblasts may mediate inflammatory and proliferative responses to injury, as occur in normal wound healing and a variety of diseases (atherosclerosis, restenosis, pulmonary inflammation (ARDS), glomerulosclerosis, etc.). A thorough understanding of how thrombin activates cells is an important goal.

A receptor that mediates thrombin signaling has been previously identified (Vu, T.-K.H. et al. (1991) Cell 64:1057-1068; USPN 5,256,766). This receptor revealed a novel proteolytic mechanism of activation and is referred to as PAR1 (protease-activated receptor 1). PAR1 is activated by the binding of thrombin to and cleavage of PAR1's amino terminal exodomain at a specific site. Receptor cleavage unmasks a new amino terminus, which then functions as a tethered peptide ligand by binding intramolecularly to the body of the receptor to effect transmembrane signaling (Vu, T.-K.H. et al. (1991) Cell 64:1057-1068). Synthetic peptides that mimic this tethered ligand domain function as PAR1 agonists and activate it independent of thrombin and receptor cleavage (Vu, T.-K.H. et al. (1991) Cell 64:1057-1068).

To identify which of thrombin's known cellular actions are mediated by PAR1, a PAR1 knockout mouse was recently generated (Connolly, A. et al. (1996) Nature 381:516-519). Analysis of mice in which both alleles of the PAR1 gene were disrupted provided definitive evidence for a second platelet thrombin receptor and for tissue specific roles of distinct thrombin receptors. Specifically, in mice, PAR1 is not important for platelet responses but is critical for fibroblast responses.

A second protease-activated receptor (PAR2) was cloned during a search for relatives of the Substance K receptor (Nystedt, S., et al. (1994) PNAS USA, 91:9208-9212). The physiological activator of PAR2 remains unknown; it is not activated by thrombin.

SUMMARY OF THE INVENTION

The protease-activated receptor (PAR3) disclosed herein is useful in assaying libraries of compounds for their activity as thrombin agonists and antagonists. DNA

encoding PAR3 is placed in a functional expression vector, expressed in a cell line, and used to assay compounds for activity as an agonist or antagonist of thrombin's affect on PAR3.

The invention features substantially pure DNA (cDNA or genomic DNA) encoding a protease-activated receptor 3 (PAR3) from vertebrate tissues (SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:4 and SEQ ID NO:5) and degenerate sequences thereof; substantially pure protease-activated receptor 3 polypeptides encoded thereby; as well as amino acid sequences substantially identical to the amino acid sequences SEQ ID NO:3 and SEQ ID NO:6 from mouse and human, respectively. The invention further comprises fragments of the PAR3 receptor which are activated by thrombin. Such fragments may have the same amino acid sequence as SEQ ID NOs:3 and 6 or be at least 80% identical to the amino acid sequences SEQ ID NO:3 and SEQ ID NO:6.

In various embodiments, the DNA, receptor or receptor fragment is derived from a vertebrate animal, preferably, human or mouse. However, the gene can be chemically synthesized.

An object of the invention is to provide a nucleotide sequence encoding a novel receptor.

Another object is to provide a cell line genetically engineered to express the nucleotide sequence.

Another object is to provide a method whereby a compound or library of compounds can be assayed as thrombin agonists or antagonists for their ability to activate or block the receptor expressed by the nucleotide sequence.

An advantage of the present invention is that a novel thrombin receptor PAR3 is disclosed making it possible to identify novel thrombin agonists and

antagonists which may not be identifiable via PAR1 or PAR2 receptors.

A feature of the invention is that it makes it possible to obtain additional information regarding thrombin activation and the sequence of biochemical events initiated by such.

These and other objects, advantages and features of the present invention will become apparent to those skilled in the art upon reading the disclosure.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is the complete nucleotide and amino acid sequences (SEQ ID NO:1 and SEQ ID NO:3, respectively) of the mouse protease-activated receptor 3 gene coding region cDNA. The deduced amino acid sequence of the receptor is provided below the nucleotide sequence and contains 369 amino acids. The deduced amino acid sequence begins at nucleotides 51-53 (ATG = Met) and ends at nucleotides 1158-1160 (TAG = stop).

Fig. 2 is the genomic sequence (containing exon 2) of the mouse protease-activated receptor 3 (SEQ ID NO:2).

Fig. 3 is the nucleotide and deduced amino acid sequences (SEQ ID NO:4 and SEQ ID NO:6, respectively) of the human protease-activated gene coding region cDNA. The deduced amino acid sequence is provided below the nucleotide sequence and contains 374 amino acids. The coding region of the cDNA sequence begins at nucleotides 58-60 (ATG = Met) and ends at nucleotides 1180-1182 (TAG = stop).

Fig. 4 is the genomic sequence (containing exon 2) of the human protease-activated receptor 3 (SEQ ID NO:5).

Fig. 5A shows the alignment of the deduced amino acid sequences (SEQ ID NO:3, 6, 7, 8, 9) of the mouse PAR3, human PAR3, human PAR1, and human PAR2. To

indicate homology, gaps (represented by blank spaces) have been introduced into the five sequences. Transmembrane domains are overlined (TM1-7). Fig. 5B shows the alignment of the hirudin-like portion of human PAR1, PAR2, and PAR3 amino acid sequences.

Fig. 6 is a bar graph showing cell surface binding of M1 monoclonal antibody to M1 epitope on Cos 7 cells expressing hPAR3 or hPAR3 T39P in the presence and absence of α-thrombin.

Fig. 7 is a bar graph of hPAR3 signaling in Cos 7 cells in the presence and absence of Gα16 and the presence and absence of α-thrombin. Signaling is measured by phosphoinositide hydrolysis.

Fig. 8 is a graph of phosphoinositide hydrolysis in response to PAR3 signaling as a function of increasing α-thrombin concentration, and in the presence and absence of Gα16 protein.

Fig. 9 is a graph of phosphoinositide hydrolysis in response to PAR3 signaling as a function of increasing γ-thrombin concentration, and in the presence and absence of Gα16 protein.

Fig. 10 is a graph comparing the specificity of PAR1 and PAR3 for thrombin.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

Before the present protease-activated receptor assays and methods of using such are described, it is to be understood that this invention is not limited to the particular DNA sequences, materials, methods, or processes described as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting since the scope

of the present invention will be limited only by the appended claims.

It must be noted that as used in this specification and the appended claims, the singular forms "a", "and," and "the" include plural referents unless the contexts clearly dictates otherwise. Thus, for example, reference to "a DNA sequence" includes mixtures and large numbers of such sequences, reference to "an assay" includes assays of the same general type, and reference to "the method" includes one or more methods or steps of the type described herein.

The publications discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the present invention is not entitled to antedate such publication by virtue of prior invention.

Unless defined otherwise, all technical and scientific terms herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials, similar or equivalent to those described herein, can be used in the practice or testing of the present invention, the preferred methods and materials are described herein. All publications cited herein are incorporated herein by reference for the purpose of disclosing and describing specific aspects of the invention for which the publication is cited in connection with.

DEFINITIONS

By "protease-activated receptor 3", "PAR3", "PAR3 receptor" and the like, is meant all or part of a vertebrate cell surface protein which is specifically

activated by thrombin or a thrombin agonist thereby activating PAR3-mediated signalling events (e.g., phosphoinositide hydrolysis, Ca^{2+} efflux, platelet aggregation). The polypeptide is characterized as having the ligand activating properties (including the agonist activating and antagonist inhibiting properties) and tissue distribution described herein. Specifically, PAR3 receptors are expressed by the DNA sequences of SEQ ID NOs:2, 4, and 5.

By a "polypeptide" is meant any chain of amino acids, regardless of length or post-translational modification (e.g., glycosylation).

By "substantially pure" is meant that the protease-activated receptor 3 polypeptide provided by the invention is at least 60%, by weight, free from the proteins and naturally-occurring organic molecules with which it is naturally associated. Preferably, the preparation is at least 75%, more preferably at least 90%, and most preferably at least 99%, by weight, PAR3 polypeptide. A substantially pure PAR3 polypeptide may be obtained, for example, by extraction from a natural source, by expression of a recombinant nucleic acid encoding a PAR3 polypeptide, or by chemically synthesizing the protein. Purity can be measured by any appropriate method, e.g., column chromatography, polyacrylamide gel electrophoresis, or HPLC analysis. The protein is substantially pure if it can be isolated to a band in a gel.

By a "substantially identical" amino acid sequence is meant an amino acid sequence which differs only by conservative amino acid substitutions, for example, substitution of one amino acid for another of the same class (e.g., valine for leucine, arginine for lysine, etc.) or by one or more non-conservative amino acid substitutions, deletions, or insertions located at

positions of the amino acid sequence which do not destroy the biological activity of the receptor. Such equivalent receptors can be isolated by extraction from the tissues or cells of any animal which naturally produce such a receptor or which can be induced to do so, using the methods described below, or their equivalent; or can be isolated by chemical synthesis; or can be isolated by standard techniques of recombinant DNA technology, e.g., by isolation of cDNA or genomic DNA encoding such a receptor. Substantially identical receptors have the same biological function, e.g. are activated by the same compound.

By "derived from" is meant encoded by the genome of that organism and present on the surface of a subset of that organism's cells.

By "isolated DNA" is meant DNA that is not in its native environment in terms of not being immediately contiguous with (i.e., covalently linked to) the complete coding sequences with which it is immediately contiguous (i.e., one at the 5' end and one at the 3' end) in the naturally-occurring genome of the organism from which the DNA of the invention is derived. The term therefore includes, for example, recombinant DNA which is incorporated into a vector; into an autonomously replicating plasmid or virus; or into the genomic DNA of a prokaryote or eukaryote; or which exists as a separate molecule (e.g., a cDNA or a genomic or cDNA fragment produced by PCR or restriction endonuclease digestion) independent of other sequences. It also includes any recombinant DNA which is part of a hybrid gene encoding additional polypeptide sequence.

"Isolated DNA" can mean the DNA is in vectors which are preferably capable of directing expression of the protein encoded by the DNA in a vector-containing cell and further includes cells containing such vectors

(preferably eukaryotic cells, e.g., CHO cells (ATCC; Cat. No. CCL 61 or COS-7 cells (ATCC; Cat. No. CRL 1651; and the *Xenopus* oocytes of the type described in the above cited reference Vu, T.-K.H. et al. (1991) Cell 64:1057-1068). Preferably, such cells are stably transfected with such isolated DNA.

By "transformed cell" and "transfected cell", "genetically engineered cell", and the like, is meant a cell into which (or into an ancestor of which) has been introduced, by means of genetic engineering, a DNA molecule encoding a PAR3 (or DNA encoding a biologically active fragment or analog, thereof). Such a DNA molecule is "positioned for expression" meaning that the DNA molecule is positioned adjacent to a DNA sequence which directs transcription and translation of the sequence (i.e., facilitates the production of the PAR3 protein, or fragment or analog, thereof).

By "specifically activates", as used herein, is meant an agent, such as thrombin, a thrombin analog, a PAR3 agonist or other chemical agent including polypeptides such as an antibody, which activates protease-activated receptor 3, receptor polypeptide or a fragment or analog thereof to initiate PAR3-mediated biological events as described herein, but which does not substantially bind other molecules in a sample, e.g., a biological sample, which naturally includes a protease-activated receptor 3 polypeptide.

By "specifically inhibits", as used herein, is meant an agent, such as a thrombin analog, a PAR3 antagonist or other chemical agent including polypeptides such as an antibody, which inhibits activation of protease-activated receptor 3, receptor polypeptide or a fragment or analog thereof, such as by inhibiting thrombin or by blocking activation of PAR3 by thrombin or other PAR3 activator. Preferably, the agent activates or

inhibits the biological activity *in vivo* or *in vitro* of the protein to which it binds.

By "biological activity" is meant the ability of the protease-activated receptor 3 to bind thrombin or a PAR3 agonist and signal the appropriate cascade of biological events (e.g., phosphoinositide hydrolysis, Ca^{2+} efflux, and platelet aggregation, and the like, as described herein.

By "substantial increase" is meant an increase in activity or other measurable phenotypic characteristic that is at least approximately a 2-fold increase over control level (where control assays are performed in the absence of activator), preferably at least approximately a 5-fold increase, more preferably at least approximately a 10-fold increase in activity over a control assay.

By "substantial decrease" or "substantial reduction" is meant a decrease or reduction in activity or other measurable phenotypic characteristic that is approximately 80% or the control level, preferably reduced to approximately 50% of the control level, or more preferably reduced to approximately 10% or less of the control level.

The terms "screening method" and "assay method" are used to describe a method of screening a candidate compound for its ability to act as an agonist of a PAR3 ligand. The method involves: a) contacting a candidate agonist compound with a recombinant protease-activated receptor 3 (or PAR3 agonist-binding fragment or analog); b) measuring activation of the receptor, the receptor polypeptide or the receptor fragment or analog; and c) identifying agonist compounds as those which interact with the recombinant receptor and trigger PAR3 activation. Interaction may be cleavage of the receptor to unmask an intramolecular receptor activating peptide or by mimicking the intramolecular receptor-activating

peptide. A tethered ligand may be more difficult to block than a free agonist. Thus, blocking thrombin is the acid test for an agonist which will block responses by other thrombin substrates.

By an "agonist" is meant a molecule which mimics a particular activity, in this case, interacting with a PAR3 ligand in a manner which activates thereby triggering the biological events which normally result from the interaction (e.g., phosphoinositide hydrolysis, Ca^{2+} efflux, and platelet aggregation). Preferably, an agonist initiates a substantial increase in receptor activity relative to control assays in the absence of activator or candidate agonist. An agonist may possess the same, less, or greater activity than a naturally-occurring PAR3 ligand.

The terms "antagonist assay", "antagonist screening" and the like, refer to a method of screening a candidate compound for its ability to antagonize interaction between a naturally-occurring activating ligand or an agonist and the PAR3. The method involves: a) contacting a candidate antagonist compound with a first compound which includes a recombinant PAR3 (or agonist-binding fragment or analog) on the one hand and with a second compound which includes thrombin or a PAR3 agonist on the other hand; b) determining whether the first and second compounds interact or are prevented from interaction by the candidate compound; and c) identifying antagonistic compounds as those which interfere with the interaction of the first compound (PAR3 receptor) to the second compound (PAR3 agonist) and which thereby substantially reduce thrombin or PAR3 agonist-activated biological events (e.g., phosphoinositide hydrolysis, Ca^{2+} efflux, and platelet aggregation).

By an "antagonist" is meant a molecule which blocks activation of a PAR3 receptor. This can be done

by inhibiting a particular activity such as the ability of thrombin, for example, to interact with a protease-activated receptor 3 thereby triggering the biological events resulting from such an interaction (e.g., phosphoinositide hydrolysis, Ca^{2+} efflux, and platelet secretion, or platelet aggregation). An antagonist may bind to and thereby block activation of a PAR3 receptor.

The terms "treatment", "treating", "treat" and the like are used herein to generally mean obtaining a desired pharmacologic and/or physiologic effect. The effect may be prophylactic in terms of completely or partially preventing a disease or symptom thereof and/or may be therapeutic in terms of a partial or complete cure for a disease and/or adverse effect attributable to the disease. "Treatment" as used herein covers any treatment of a disease in a mammal, particular a human, and includes:

(a) preventing the disease or symptom from occurring in a subject which may be predisposed to the disease or symptom but has not yet been diagnosed as having it;

(b) inhibiting the disease symptom, i.e., arresting its development; or

(c) relieving the disease symptom, i.e., causing regression of the disease.

PREFERRED EMBODIMENTS

In preferred embodiments of both screening methods, the recombinant PAR3 is stably expressed by a vertebrate cell which normally presents substantially no PAR3 on its surface (i.e., a cell which does not exhibit any significant thrombin-mediated phosphoinositide hydrolysis or Ca^{2+} efflux in the presence of a PAR activator); the vertebrate cell is a mammalian cell, is a

Rat 1 cell, or a COS 7 cell; and the candidate antagonist or candidate agonist is a thrombin analog, PAR3 peptide fragment or analog or other chemical agent including a polypeptide such as an antibody.

The receptor proteins of the invention are likely involved in the activation of vertebrate platelet, leukocyte, and mesenchymal cells in response to wounding, as well as mediating signalling in embryonic development. Such proteins and in particular PAR3 antagonists are useful therapeutics for the treatment of such conditions as thrombosis, atherosclerosis, restenosis, and inflammation associated with normal wound healing and a variety of diseases including atherosclerosis, restenosis, pulmonary inflammation (ARDS) and glomerulosclerosis. Preferred therapeutics include 1) agonists, e.g., thrombin analogs, PAR3 peptide fragments or analogs thereof, or other compounds which mimic the action of thrombin upon interaction with the protease-activated receptor 3 or mimic the action of an intramolecular receptor activating peptide; and 2) antagonists, e.g., thrombin analogs, antibodies, or other compounds, which block thrombin or protease-activated receptor 3 function by interfering with the thrombin:receptor interaction or by interfering with the receptor intramolecular activating peptide. The dosage would be expected to be comparable with current antiflammatory drugs and should be adjusted based on the age, sex, weight and condition of the patient beginning with small doses and increasing gradually based on responsiveness and toxicity.

Because the receptor component may now be produced by recombinant techniques and because candidate agonists and antagonists may be screened using transformed, cultured cells, the instant invention provides a simple and rapid approach to the identification of useful

therapeutics. Isolation of the PAR3 gene (as cDNA or genomic DNA) allows its expression in a cell type which does not normally bear PAR3 on its surface, providing a system for assaying a thrombin:receptor interaction and receptor activation.

EXAMPLES

The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make receptor proteins and sequences encoding such proteins and carry out the methodology for finding such DNA sequences and proteins, and are not intended to limit the scope of what is regarded as the invention. Efforts have been made to insure accuracy with respect to numbers used (e.g., amounts, temperature, etc.) but some experimental errors and deviations should be accounted for. Unless indicated otherwise, parts or parts by weight, molecular weight is weight average molecular weight; temperature is in degrees centigrade; and pressure is at or near atmospheric.

There now follows a description of the cloning and characterization of the cDNA, genomic DNA and the receptor protein of the protease-activated receptor 3 from mouse and human. Expression vectors containing and capable of expressing the PAR3 DNA, as well as transformed cells containing and expressing the DNA of the invention are also described. Also described are possible PAR3 agonists and antagonists as well as screening assays for receptor agonists and receptor antagonists.

EXAMPLE 1

Isolation of the Mouse Protease-Activated Receptor 3

Rat platelets were used as a source of RNA in the search for and cloning of PAR3 because rat platelets are more abundant than mouse platelets and, like mouse platelets, they do not respond to PAR1 agonist peptides (Connolly, A. et al. (1996) Nature 381: 516-519; and Connolly, T.M. et al (1994) Thromb Haemost 72: 627-33).

Total RNA was prepared from rat platelets using Trizol reagent (Gibco BRL). cDNA was then prepared using random hexamer primers and the Superscript reverse transcriptase system (Gibco, BRL). cDNA was then used as template for PCR amplification using a Robocycler Gradient 96® (Stratagene) and the primers 5'-GTITACATGCTI(A/C)AC(C/T)TIGCI(A/C/G/T)TIGC(A/C/G/T)GA-3' (SEQ ID NO:10) and 5'-GGATAIACIACIGCIA(A/G/T)(A/G)(A/T)AIC(G/T)(A/C/G/T)TC-3' (SEQ ID NO:11) at 5μM in 20μM Tris-HCl (pH 8.4), 50μM KCl, 1.5μM MgCl2, 0.2μM dNTP, and 50U/μl Taq polymerase. Polymerase chain reaction temperature was varied as follows: 94°C for 4 min; 30 cycles of 94°C for 45 sec, 39°C for 60 sec, and 72°C for 90 sec; then 72°C for 7 min. PCR products were subcloned using the TA cloning kit (InVitrogen, San Diego, CA). Rat cDNA clones with inserts of approximately 200 bp were analyzed by nucleic acid sequencing. One sequence predicted a novel G-protein coupled receptor related to PAR1 and PAR2. This sequence was used to obtain mouse and human cDNA and genomic clones by a combination of PCR and hybridization techniques (see, for example, Sambrook, J. et al. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York). The nucleotide sequences are shown in Figures 1-4.

The rat PCR product was then used to clone the full length mouse cDNA and genomic DNA clones. The nucleotide sequences and deduced amino acid sequence of the mouse PAR3 are shown in Figs. 1 and 2.

The human PAR3 cDNA used for the functional studies presented below was cloned from a Lamda gt 10 intestinal cDNA library (Clonetech). Features of human PAR3's amino acid sequence are shown in Figs. 5A and 5B by alignment of the deduced amino acid sequence of PAR3 with those of PAR1 and PAR2. Predicted transmembrane (TM) domains are overlined and predicted Asn-linked glycosylation sites in PAR3 are underlined in the figure. The amino terminal exodomains are compared in Fig. 5b, including the cleavage site (^), the tethered ligand domains of PAR1 and PAR2, and the predicted tethered ligand domain of PAR3 (underlined). Also underlined is PAR3's hirudin-like domain (FEEFP). The similar FEEIP and YEPFW sequences in hirudin and PAR1, respectively are known to bind thrombin's fibrinogen-binding exosite.

The human PAR3 cDNA contained an open reading frame encoding a 374 amino acid putative G protein-coupled receptor (Fig. 3). BLAST search of the Genbank and EST databases revealed this protein to be novel with 28% and 30% amino acid sequence identity to human PAR1 and PAR2 (Fig. 5a, Table I). Its amino terminal exodomain revealed a possible thrombin cleavage site and a striking hirudin-like sequence (Fig. 5b). Like the carboxyl tail of hirudin itself, PAR1's hirudin-like sequence is known to dock with thrombin's fibrinogen binding exosite, an interaction important for efficient PAR1 cleavage by thrombin (Vu, T.-K.H. et al.(1991) Nature 353:674-677; Liu, L. et al. (1991) J. Biol. Chem 266:16977-16980; Mathews, I.I. et al. (1994) Biochem 33 3266-79; Ishii, K. (1995) J. Biol. Chem 270:16435-16440, which references are herein incorporated by reference in

their entirety). These observations strongly suggested that this new receptor was a novel thrombin receptor.

A comparison of PAR deduced amino acid sequences from human, mouse, and *Xenopus* is provided in Table I below. The % identity of the total sequence as well as the % identity of the transmembrane regions are shown.

TABLE I

PAR SEQUENCE	% AMINO ACID IDENTITY	
	TOTAL	TM1-7
hPAR3 vs hPAR1	28	37
hPAR3 vs hPAR2	30	38
hPAR1 vs hPAR2	28	42
hPAR3 vs xPAR1	29	38
hPAR1 vs xPAR1	52	63
hPAR3 vs mPAR3	67	74
hPAR1 vs mPAR1	77	81
hPAR2 vs mPAR2	78	85

h = human m = mouse x = Xenopus laevis

EXAMPLE 2

Polypeptide Expression

Polypeptides according to the invention may be produced by transformation of a suitable host cell with all or part of a PAR3 encoding cDNA fragment (e.g., the cDNAs described above) in a suitable expression vehicle, and expression of the receptor.

Those skilled in the field of molecular biology will understand that any of a wide variety of expression systems may be used to provide the recombinant receptor protein. The precise host cell used is not critical to the invention. The receptor may be produced in a prokaryotic host (e.g., *E. coli*) or in a eukaryotic host (e.g., *Saccharomyces cerevisiae* or mammalian cells, e.g., COS-6M, COS-7, NIH/3T3, or Chinese Hamster Ovary cells). Such cells are available from a wide range of sources (e.g., the American Type Culture Collection, Rockville, MD). The method of transfection and the choice of expression vehicle will depend on the host system selected. Transformation and mammalian cell transfection methods are described, e.g., in Ausubel et al. (Current Protocols in Molecular Biology, John Wiley & Sons, New York, (1989)); expression vehicles may be chosen from those provided, e.g., in Cloning Vectors: A Laboratory Manual (Pouwels, P.H. et al., (1985), Supp. 1987).

Particularly preferred expression systems are the *Xenopus* oocyte cells of Vu et al. (Vu et al., Cell (1991) *supra*) and insect cells (SF9-baculovirus) transfected with an expression vector containing and expressing a receptor protein or biologically active fragment thereof. DNA encoding the human or mouse PAR3 or an appropriate receptor fragment or analog (as described above) is inserted into the expression vector in an orientation designed to allow expression. Alternatively, the PAR3 (or biologically active receptor fragment or analog) is expressed by a stably-transfected mammalian cell line. Other preferable host cells which may be used in conjunction with the expression vehicle include NIH/3T3 cells (ATCC Accession No. 1658). The expression may be used in a screening method of the invention (described below) or, if desired, the recombinant receptor protein may be isolated as described below.

A number of vectors suitable for stable transfection of mammalian cells are available to the public, e.g., see Pouwels et al. (supra); methods for constructing such cell lines are also publicly available, e.g., in Ausubel et al. (supra). In one example, cDNA encoding the receptor (or receptor fragment or analog) is cloned into an expression vector which includes the dihydrofolate reductase (DHFR) gene. Integration of the plasmid and, therefore, the PAR3-encoding gene into the host cell chromosome is selected for by inclusion of 0.01-300 μM methotrexate in the cell culture medium (as described in Ausubel et al., supra). This dominant selection can be accomplished in most cell types. Recombinant protein expression can be increased by DHFR-mediated amplification of the transfected gene. Methods for selecting cell lines bearing gene amplifications are described in Ausubel et al. (supra); such methods generally involve extended culture in medium containing gradually increasing levels of methotrexate. DHFR-containing expression vectors commonly used for this purpose include pCVSEII-DHFR and pAdD26SV(A) (described in Ausubel et al., supra). Any of the host cells described above or, preferably, a DHFR-deficient CHO cell line (e.g., CHO DHFR cells, ATCC Accession No. CRL 9096) are among the host cells preferred for DHFR selection of a stably-transfected cell line or DHFR-mediated gene amplification.

One particularly preferred stable expression system is a Rat 1 cell (ATCC) stably transfected with a pcDNAI/NEO (InVitrogen, San Diego, CA) expression vector.

Expression of the recombinant receptor (e.g., produced by any of the expression systems described herein) may be assayed by immunological procedures, such as Western blot or immunoprecipitation analysis of recombinant cell extracts, or by immunofluorescence of

intact recombinant cells (using, e.g, the methods described in Ausubel et al., supra). Recombinant receptor protein is detected using an antibody directed to the receptor. Described below are methods for producing anti-protease-activated receptor 3 antibodies using, as an immunogen, the intact receptor or a peptide which includes a suitable protease-activate receptor 3 epitope. To detect expression of a PAR3 fragment or analog, the antibody is preferably produced using, as an immunogen, an epitope included in the fragment or analog.

Once the recombinant PAR3 protein (or fragment or analog, thereof) is expressed, it is isolated, e.g., using immunoaffinity chromatography. In one example, an anti-PAR3 antibody may be attached to a column and used to isolate intact receptor or receptor fragments or analogs. Lysis and fractionation of receptor-harboring cells prior to affinity chromatography may be performed by standard methods (see, e.g., Ausubel et al., supra). Once isolated, the recombinant protein can, if desired, be further purified, e.g., by high performance liquid chromatography (see, e.g., Fisher, Laboratory Techniques In Biochemistry And Molecular Biology, eds., Work and Burdon, Elsevier, (1980)).

Receptors of the invention, particularly short receptor fragments, can also be produced by chemical synthesis (e.g., by the methods described in Solid Phase Peptide Synthesis, (1984) 2nd ed., The Pierce Chemical Co., Rockford, IL).

EXAMPLE 3

Cleavage and Activation Studies of the Recombinant Protease-Activated Receptor 3

PAR3 was demonstrated to be a substrate for thrombin when expressed on the surface of Cos 7 cells (Fig. 6). Human PAR1 or PAR3 cDNAs that were modified to

encode receptors displaying a FLAG epitope (amino acid sequence DYKDDD (SEQ ID NO:12) at a site amino to the thrombin cleavage site were transiently expressed in Cos7 cells. Epitope-tagged PAR1 has been previously described (Ishii, K. et al. (1993) J. Biol. Chem. 268:9780-9786). The analogous epitope-tagged PAR3 cDNA was constructed so as to encode a new amino terminus with the sequence MDSKGSSQKGSRLLLLLVVSNLLLCQGVVS/DYKDDDDVE-TF (SEQ ID NO:13) representing the prolactin signal peptide, putative signal peptidase site (/), FLAG epitope DYKDDDD (SEQ ID NO:12) and junction VE fused to amino acid 17 in PAR 3.

cDNAs were subcloned into the mammalian expression vector pBJ1. For receptor cleavage studies Cos 7 cells were transfected using DEAE-dextran and thrombin-mediated loss of M1 antibody (Kodak) binding to the FLAG epitope of the cell surface using a procedure described by Ishii et al. (Ishii, K. et al. (1993) *supra*). Over 95% of M1 antibody binding was transfection-dependent in this system. Cells were incubated for 5 min. at 37°C in the presence (open columns) or absence (closed columns) of 20nM thrombin (Fig. 6). For biochemical identification of the cleavage site, cleavage of soluble PAR3 amino terminal exodomain by thrombin was assayed as follows. A recombinant PAR3 soluble exodomain was prepared in which the amino terminal exodomain residues 21-94 were sandwiched between a translational start and hexahistidine tag (i.e. MG-[*PAR3* 21-94]-VEHHHHHH; where VEHHHHHH is SEQ ID NO:18). The recombinant protein was expressed as a soluble polypeptide in *E. coli*, purified, and analyzed before and after thrombin cleavage as previously described for the analogous region of PAR1 (Ishii, K. (1995) J. Biol. Chem. 270:16435-16440). Recombinant soluble amino terminal exodomain was cleaved in solution with 50nM thrombin for 1h at 37°C, then

analyzed by SDS-PAGE. Even prolonged incubation with a high concentration of thrombin yielded only one detectable cleavage event indicating that only one thrombin cleavage site exists in the PAR3 exodomain. Amino acid sequencing of the cleavage products revealed only a single new amino terminus with the sequence TFRG (see Fig. 1b). Thus, thrombin recognizes and cleaves PAR3 in the amino terminal exodomain between amino acids K38 and T39 with high specificity.

EXAMPLE 4

PAR3 Signaling Activity

The ability of PAR3 to mediate signaling by thrombin was tested. *Xenopus* oocytes were microinjected with cRNA encoding epitope-tagged human PAR3 (hPAR3), hPAR3 bearing the T39P cleavage site mutation, or the F40A tethered ligand domain mutation. Thrombin-triggered ^{45}Ca release was measured as described in Vu et al. (Vu, T.-K. H. et al. (1991) *supra*). Surface expression of wild type and mutant receptors was confirmed by M1 antibody binding by the method of Ishii, K. et al. (Ishii, K. et al. (1995) J. Biol. Chem. 270:16435-16440; and Ishii, K. et al. (1993) J. Biol. Chem. 268:9780-9786, which references are herein incorporated by reference in their entirety).

Microinjection of *Xenopus* oocytes with human PAR3 cRNA conferred thrombin-dependent ^{45}Ca mobilization (Fig. 7) which reflects agonist-triggered phosphoinositide hydrolysis in this system. Mutation of PAR3's thrombin cleavage site ablated thrombin signaling and thrombin rendered proteolytically inactive by the active site inhibitor PPACK failed to activate PAR3 even at concentrations as high as 1μM. These data strongly

suggest that cleavage of the K 38 -T 39 peptide bond is necessary for PAR3 activation by thrombin.

The specificity of PAR3 and PAR1 signaling was also examined. Protease-triggered ^{45}Ca release was measured in *Xenopus* oocytes expressing human PAR1 or PAR3 stimulated with various concentrations of the arginine/lysine specific serine proteases trypsin, Factor Xa, Factor VIIa, tissue plasminogen activator, or plasmin. Chymotrypsin, elastase, and cathepsin G were also tested. PAR3 was at least as specific for thrombin as thrombin receptor PAR1 (Fig. 10).

PAR3 signaling in Cos 7 cells was also examined. Cos 7 cells were transfected with human PAR1 or PAR3. Cells were then metabolically labelled with ^{3}H-inositol and phosphoinositide hydrolysis was measured in response to the indicated concentrations of α-thrombin (Fig. 8) or γ-thrombin (Fig. 9) as described by Ishii, et al. and Nanevicz et al. (Ishii, K. et al. (1993) *supra*; and Nanevicz, T. et al. (1996) J. Biol. Chem. 271:702-706).

Co-transfection with α16, a G protein α-subunit expressed in hematopoietic cell lines (Amatruda III, T.T. et al. (1991) J. Biol. Chem. 266:5587-5591) caused a 50-150% increase in the maximal PAR3-mediated response to thrombin in these cells in each of three separate experiments (Fig. 7).

The EC_{50} for thrombin signaling through PAR3 in this system was approximately 0.2 nM, comparable to that seen with PAR1 and well within physiologically achievable thrombin concentrations (Fig 8). γ-thrombin, which is defective in its anion-binding exosite (Rydel, T.J. et al. (1994) J. Biol. Chem. 269:22000-22006), was two log units less potent than α-thrombin (EC_{50} = 20nM; Fig. 9). Similarly, incubation of α-thrombin with the fibrinogen binding exosite blocker hirugen (Skrzypczak, J.E. et al. (1991) J. Mol. Biol. 221:1379-1393) right-shifted the

dose response curve two logs (not shown). Alanine substitution at F 48 and E 49 in PAR3's hirudin-like sequence, residues predicted to dock with thrombin's fibrinogen-binding exosite by analogy with hirudin and PAR1 (Fig. 5B) also caused a decrease in thrombin signaling by PAR3. These data strongly suggest that PAR3 interacts with thrombin in a manner similar to PAR1 (Mathews, I. I., et al. (1994) Biochem. 33:3266-3279). Specifically, it is likely that PAR3 amino acids 48-52 (FEEFP, SEQ ID NO:14) dock with thrombin's fibrinogen-binding exosite while amino acids 35-38 (LTPK, SEQ ID NO:15) dock with thrombin's active center leading to cleavage of the K 38 - T 39 peptide bond.

Synthetic peptides that mimic the new amino terminus unmasked by receptor proteolysis, the so called "tethered ligand domain", act as agonists for PAR1 and PAR2 (Vu, T. K.-H. et al. (1991) Cell 64:1057-1068; Nystedt, S. et al. (1994) PNAS USA 91:9208-9212; and USPN 5,256,766, which references are herein incorporated by reference in their entirety).

Peptides homologous to the tethered domain of PAR3 may be tested as potential agonists of PAR3 activity. Two peptides, TFRGAP (SEQ ID NO:16) and TFRGAPPNS (SEQ ID NO:17) were synthesized and tested for their ability to mimic the action of thrombin by causing PAR3 signaling as measured by phosphoinositide hydrolysis. Cos 7 cells expressing human PAR3 were incubated with the peptides at concentrations up to 100μM. Phosphoinositide hydrolysis was not observed to be above control levels indicating that the synthetic peptides caused no detectable signaling by PAR3 under these conditions, whereas an EC_{50} of 0.2 nM was determined for α-thrombin under the same assay conditions. These results demonstrate that monitoring phosphoinositide hydrolysis provides a useful

means for assessing potential agonists for activity on PAR3 signaling for use as potential pharmaceutics.

The tethered ligand domain of PAR3 was required for PAR3 activation by thrombin. Substitution of Ala for Phe 40 (the F40A PAR3 mutant), which is analogous to the critical Phe 43 in PAR1's tethered ligand (Scarborough, R.M. et al. (1992) J. Biol. Chem. 267:13146-13149), ablated PAR3 signaling but not PAR3 cleavage by thrombin. The observation that cleavage of the Lys 38-Thr 39 peptide bond is necessary for PAR3 activation suggests that PAR3 is probably activated by the same tethered ligand mechanism utilized by PAR1 and 2.

EXAMPLE 5

PAR3 Tissue Expression in Mouse and Human

In situ hybridization of mouse tissue revealed the presence of PAR3 mRNA in megakaryocytes in mouse spleen. In the tissues examined (brain, eye, thymus, heart, lung, liver spleen, pancreas, stomach, small intestine, colon, kidneys, bladder, uterus, ovary, testis, skeletal muscle, peripheral nerve, and skin), megakaryocytes in the spleen were the only cells which displayed clearcut hybridization over background. Control samples in which hybridization was performed with a sense strand probe control were negative for all cells. Northern analysis of mouse tissues for PAR3 mRNA showed signals in spleen and lung, with low levels seen in brain, heart, and other tissues. Spleen is a hematopoietic organ in mouse, and megakaryocytes are sometimes seen trapped in the pulmonary microvasculature. Thus both Northern and *in situ* hybridization data suggest that PAR3 is most abundantly expressed in megakaryocytes in the mouse.

The pharmacology of hPAR3 activation in Cos cells resembles that of mouse platelet activation. Both responses show subnanomolar EC_{50}s for activation by α-thrombin and are thrombin active site- and fibrinogen-binding exosite-dependent. These observations support the concept that the mouse homolog of PAR3 is a thrombin receptor that mediates thrombin responses in mouse platelets. Whether human PAR3 function in human platelets remains to be determined.

The *in situ* hybridization studies were performed as follows. Anesthetized adult C57BL/6 mice were perfusion-fixed with 4% paraformaldehyde. Organs to be tested were dissected, trimmed, and immersion-fixed for 4 hours in 4% paraformaldehyde. Processed tissues were embedded in paraffin, and 5 mm sections were cut. Sense or antisense ^{35}S-riboprobe was transcribed *in vitro* from mouse PAR2 cDNA subcloned into the *Eco*R1 site of pBluescript II SK$^-$ (Stratagene, San Diego, CA). Hybridization, wash, and development conditions were as reported for mouse PAR1 (Soifer, S.J. et al. (1993) Am. J. Pathol. 144:60-69). To carry out Northern analysis a ^{32}P-labeled probe for the mouse message was generated by random priming (Prime-It II kit; Stratagene) of PCR-amplified DNA fragments corresponding to mouse cDNA codons representing transmembrane domains 2 to 3. High stringency hybridizations and washes were performed as per the Clontech protocol for Northern analysis.

Northern analysis of human tissues revealed that PAR3 mRNA is widely distributed with signals noted in small intestine, bone marrow, heart, pancreas, lung, liver, adrenal, trachea, lymph node, stomach, and peripheral blood leukocytes. The role of PAR3 in these various human tissues awaits definition; the finding of PAR3 in human bone marrow and leukocytes is consistent

with PAR3's playing a role in mediating activation of platelets and other hematopoietic cells by thrombin.

EXAMPLE 6

Assays for Protease-Activated Receptor 3 Function

Useful receptor fragments or analogs of the invention are those which interact with thrombin and are activated to initiate the cascade of events associated with thrombin:receptor interaction. Such an interaction may be detected by an *in vitro* functional assay method (e.g., the phosphoinositide hydrolysis assay, ^{45}Ca efflux assay, or platelet aggregation assay described herein). This method includes, as components, thrombin and a recombinant protease-activated receptor 3 (or a suitable fragment or analog) configured to permit thrombin binding (e.g., those polypeptides described herein). Thrombin may be obtained from Sigma Chemical Co. (St. Louis, MO) or similar supplier.

Preferably, the protease-activated receptor 3 component is produced by a cell that naturally presents substantially no receptor on its surface, e.g., by engineering such a cell to contain nucleic acid encoding the receptor component in an appropriate expression system. Suitable cells are, e.g., those discussed above with respect to the production of recombinant receptor, such as Rat 1 cells or COS-7 cells.

EXAMPLE 7

Screening For Protease-Activated Receptor 3 Activator Antagonists and Agonists

Antagonists

As discussed above, one aspect of the invention features screening for compounds that inhibit the interaction between thrombin (or other PAR3 activating

compound) and the protease-activated receptor 3, thereby preventing or reducing the cascade of events that are mediated by that interaction. The elements of the screen are a PAR3 activator (such as thrombin), a candidate antagonist, and recombinant PAR3 (or a suitable receptor fragment or analog, as outlined above) configured to permit detection of PAR3 activator, antagonist, and PAR3 function. An additional element may be a downstream substrate, such as phosphoinositide, the hydrolysis of which is used to measure thrombin activity (Ishii, K. et al. (1993) *supra;* and Nanevicz, T. et al. (1996) *supra*).

Inhibition of thrombin-induced platelet aggregation may also be used as a means of monitoring an antagonist of PAR3 receptor activation. Thrombin is incubated with the candidate inhibitory compound (such as a peptide) for 5 minutes, then the mixture is added to washed platelets and platelet activation is followed as platelet ATP secretion by lumiaggregometry (see, for example, Connolly, A.J. et al. Nature 381:516-519 (1996); and USPN 5,256,766). Alternately, platelets are incubated with a candidate PAR 3 antagonist for 5 minutes. Thereafter the response to thrombin is measured.

Inclusion of potential antagonists in the screening assay along with thrombin allows for the screening and identification of authentic receptor antagonists as those which decrease thrombin-mediated events, such as platelet aggregation.

Appropriate candidate thrombin antagonists include PAR3 fragments, particularly, fragments of the protein predicted to be extracellular and therefore likely to bind thrombin or the tethered ligand; such fragments would preferably include five or more amino acids.

Candidate PAR 3 antagonists include thrombin analogs as well as other peptide and non-peptide compounds and anti-PAR3 antibodies.

AGONISTS

Another aspect of the invention features screening for compounds that act as PAR3 ligand agonists. Activation of the PAR3 with thrombin or an agonist leads to a cascade of events (such as phosphoinositide hydrolysis, Ca^{2+} efflux, and platelet aggregation), providing a convenient means for measuring thrombin or other agonist activity.

The agonist screening assay of the invention utilizes recombinant cells expressing recombinant PAR3 (or a suitable receptor fragment or analog, as outlined herein) configured to permit detection of PAR3 function. Alternatively, a cell such as a leukocyte, a platelet, or a mesenchymal cell that naturally expresses PAR3 may be used. Other elements of the screen include a detectable downstream substrate of the PAR3 activation, such as radiolabelled phosphoinositide, the hydrolysis of which to a detectable product indicates PAR3 activation by the candidate agonist.

^{45}Ca efflux from a cell expressing PAR3 may be used as a means of measuring receptor activation by candidate agonists (Williams, J.A. et al., (1988) PNAS USA 85:4939-4943; Vu, T.-K. H., et al. (1991) Cell 64:1057-1068; and USPN 5,256,766, which references are herein incorporated by reference in their entirety). ^{45}Ca release by oocytes expressing cRNA encoding PAR3 are assessed as follows. Briefly, intracellular calcium pools are labeled by incubating groups of 30 oocytes in 300 μl calcium-free MBSH containing 50 μCi $^{45}CaCl_2$ (10-40 mCi/mg Ca; Amersham) for 4 hours at room temperature.

The labeled oocytes are washed, then incubated in MBSH II without antibiotics for 90 minutes. Groups of 5 oocytes are selected and placed in individual wells in a 24-well tissue culture plate (Falcon 3047) containing 0.5 ml/well MBSH II without antibiotics. This medium is removed and replaced with fresh medium every 10 minutes, the harvested medium is analyzed by scintillation counting to determine ^{45}Ca released by the oocytes during each 10-minute incubation. The 10-minute incubations are continued until a stable baseline of ^{45}Ca release per unit time is achieved. Two additional 10-minute collections are obtained, then test medium including agonist is added and agonist-induced ^{45}Ca release determined.

A voltage clamp assay provides an alternative method of monitoring agonist activity. Agonist-induced inward chloride currents are measured in voltage-clamped oocytes expressing thrombin receptor encoding cRNA essentially as previously described (Julius, D. et al. Science (1988) 241:558-563, herein incorporated by reference in its entirety) except that the single electrode voltage-clamp technique is employed.

Platelet aggregation may also be used as a means of monitoring PAR3 receptor activation (see, for example, Connolly, A.J. et al. Nature 381:516-519 (1996). In particular, mouse platelets may utilize only PAR 3 for thrombin signaling. Human platelets may use both PAR 1 and PAR 3. Thus both would be useful in deleting against function at PAR 3.

An agonist useful in the invention is one which imitates the normal thrombin-mediated signal transduction pathway leading, e.g., to an increase in phosphoinositide hydrolysis. Appropriate candidate agonists include thrombin analogs or PAR3 tethered ligand domains or other agents which mimic the action of thrombin or the PAR 3 tethered ligand domain. Agonists would be useful for aiding discovery of antagonists.

EXAMPLE 8

Anti-Protease-Activated Receptor 3 Antibodies

Protease-activated receptor 3 (or immunogenic receptor fragments or analogs) may be used to raise antibodies useful in the invention. Receptor fragments preferred for the production of antibodies are those fragments deduced or shown experimentally to be extracellular.

Antibodies directed to PAR3 peptides are produced as follows. Peptides corresponding to all or part of the PAR3 protein are produced using a peptide synthesizer by standard techniques. The peptides are coupled to KLH with m-maleimide benzoic acid N-hydroxysuccinimide ester. The KLH-peptide is mixed with Freund's adjuvant and injected into animals, e.g. guinea pigs or goats, to produce polyclonal antibodies. Monoclonal antibodies may be prepared using the PAR3 polypeptides described above and standard hybridoma technology (see, e.g., Kohler et al., Nature (1975) 256:495, 1975; Kohler et al., Eur. J. Immunol. (1976) 6:292; Kohler et al., Eur. J. Immunol. (1976) 6:511; Hammerling et al., in Monoclonal Antibodies and T Cell Hybridomas, Elsevier, NY, (1981); and Ausubel et al., supra). Antibodies are purified by peptide antigen affinity chromatography.

Once produced, antibodies are tested for their ability to bind PAR3 by specific binding to the surface of PAR3-transfected cells by Western blot or immunoprecipitation analysis (such as by the methods described in Ausubel et al., supra).

Antibodies which specifically recognize PAR3 are considered to be likely candidates for useful antagonists; such candidates are further tested for their ability to specifically interfere with the interaction between thrombin and PAR3 (using the functional antagonist assays described herein). Antibodies which antagonize thrombin:PAR3 binding or PAR3 function are considered to be useful antagonists in the invention.

EXAMPLE 9

THERAPY

Particularly suitable therapeutics for the treatment of wound healing, thrombosis, atherosclerosis, restenosis, inflammation, and other thrombin-mediated signalling disorders are the agonists and antagonists described above formulated in an appropriate buffer such as physiological saline. Where it is particularly desirable to mimic a receptor fragment conformation at the membrane interface, the fragment may include a sufficient number of adjacent transmembrane residues. In this case, the fragment may be associated with an appropriate lipid fraction (e.g., in lipid vesicles or attached to fragments obtained by disrupting a cell membrane). Alternatively, anti-PAR3 antibodies produced as described above may be used as a therapeutic. Again, the antibodies would be administered in a pharmaceutically-acceptable buffer (e.g., physiological saline). If appropriate, the antibody preparation may be combined with a suitable adjuvant.

Antibodies to PAR 3 are useful antagonists which can be formulated as indicated above. Other therapeutically useful antagonists are peptides derived from PAR3 that bind to and block thrombin and include formulation comprising a pharmaceutically acceptable carrier and one or more of the following:

(1) the isolated sequence LPIKTFRGAPPNSFEEFPFSALE;

(2) uncleavable thrombin inhibitor LPIKPFRGAPPNSFEEFPFSALE where the PAR 3 cleavage site P1' is mutated to block cleavage;

(3) uncleavable thrombin inhibitor LPI (hR) TFRGAPPNSFEEFPFSALE where the PAR 3 cleavage site P1 is mutated to block cleavage;
hR is beta-homoarginine (the extra methylene group is in the main chain);

(4) uncleavable thrombin inhibitor (dF) PRPFRGAPPNSFEEFPFSALE where the good active site binding sequence dFPR is substituted for LPIK; dF is D-Phenylalanine;

(5) any of (1)-(4) above where all or part of the sequence TFRGAPPNS is replaced with spacer sequences such as GGG;

(6) variations and combinations of (1)-(5) which act as antagonists.

The therapeutic preparation is administered in accordance with the condition to be treated. Ordinarily, it will be administered intravenously, at a dosage, of a duration, and with the appropriate timing to elicit the desired response. Appropriate timing refers to, for example, time relative to wounding, time intervals between therapeutic administrations, and the like, at

which administration of therapeutic preparation elicits the desired response. Alternatively, it may be convenient to administer the therapeutic orally, nasally, or topically, e.g., as a liquid or a spray. The dosages are determined to be an amount of the therapeutic agent delivered to an animal that substantially reduces or alleviates disease symptoms. Treatment may be repeated as necessary for substantial reduction or alleviation of disease symptoms.

PAR3 activator agonists can be used for the treatment of bleeding. Antagonists may be useful in controlling the formation of clots that cause heart attack and stroke, mediating inflammation and the proliferative responses to injury in normal wound healing and a variety of diseases including atherosclerosis, restenosis, pulmonary inflammations (ARDS), glomerulosclerosis, etc.

The methods of the invention may be used to screen therapeutic receptor activator agonists and antagonists for their effectiveness in altering thrombin-mediated biological events, such as phosphoinositide hydrolysis or other cell signalling events by the assays described above. Where a non-human mammal is treated or where a therapeutic for a non-human animal is screened, the PAR3 or receptor fragment or analog or the antibody employed is preferably specific for that species.

OTHER EMBODIMENTS

Polypeptides according to the invention include any protease-activated receptors (as described herein). Such receptors may be derived from any source, but are preferably derived from a vertebrate animal, e.g., a human or mouse. These polypeptides are used, e.g., to

screen for antagonists which disrupt, or agonists which mimic, a thrombin:receptor interaction.

Polypeptides of the invention also include any analog or fragment of a PAR3 capable of interacting with thrombin. Such analogs and fragments may also be used to screen for PAR3 ligand antagonists or agonists. In addition, that subset of receptor fragments or analogs which bind thrombin and are, preferably, soluble (or insoluble and formulated in a lipid vesicle) may be used as antagonists to reduce the *in vivo* concentration of endogenous thrombin, either circulating concentration or local concentration. The efficacy of a receptor analog or fragment is dependent upon its ability to interact with thrombin; such an interaction may be readily assayed using PAR3 functional assays (e.g., those described herein).

Specific receptor analogs of interest include full-length or partial receptor proteins including an amino acid sequence which differs only by conservative amino acid substitutions, for example, substitution of one amino acid for another of the same class (e.g., valine for leucine, arginine for lysine, etc.) or by one or more non-conservative amino acid substitutions, deletions, or insertions located at positions of the amino acid sequence which do not destroy the receptors' ability to signal thrombin-mediated events (e.g., as assayed above).

Specific receptor fragments of interest include any portion of the PAR3 which is capable of interacting with thrombin, for example, all or part of the extracellular domains predicted from the deduced amino acid sequence. Such fragments may be useful as antagonists (as described above), and are also useful as immunogens for producing antibodies which neutralize the activity of PAR3 *in vivo* (e.g., by interfering with the

interaction between the receptor and thrombin). The sequence of figure 5B is most likely to bind thrombin. Modification of the (K38/T39) cleavage site for example, substitution of proline for T39 will render peptides mimicking this site uncleavable. Such peptides will bind thrombin with high affinity.

Extracellular regions of novel protease-activated receptors may be identified by comparison with related proteins of similar structure (e.g., other members of the G-protein-coupled receptor family); useful regions are those exhibiting homology to the extracellular domains of well-characterized members of the family.

Alternatively, from the primary amino acid sequence, the secondary protein structure and, therefore, the extracellular domain regions may be deduced semi-empirically using a hydrophobicity/hydrophilicity calculation such as the Chou-Fasman method (see, e.g., Chou and Fasman, Ann. Rev. Biochem. (1978) 47:251). Hydrophilic domains, particularly ones surrounded by hydrophobic stretches (e.g., transmembrane domains) present themselves as strong candidates for extracellular domains. Finally, extracellular domains may be identified experimentally using standard enzymatic digest analysis, e.g., tryptic digest analysis.

Candidate fragments (e.g., any extracellular fragment) are tested for interaction with thrombin by the assays described herein (e.g., the assay described above). Such fragments are also tested for their ability to antagonize the interaction between thrombin and its endogenous receptor, such as PAR3, using the assays described herein. Analogs of useful receptor fragments (as described above) may also be produced and tested for efficacy as screening components or antagonists (using the assays described herein); such analogs are also considered to be useful in the invention.

Identification of the receptor(s) that mediate thrombin signaling provides potential targets for the development of drugs that block thrombin's undesirable actions or mimic its desirable activities. Thrombin receptor antagonists may be used for inhibition of platelet-dependent thrombosis in the setting of unstable angina and myocardial infarction or for blocking thrombin's proinflammatory actions on endothelial cells in the setting of vascular injury. Thrombin receptor agonists may be used to promote hemostasis and fibroblast proliferation at wound sites.

Unmasked tethered ligand domain peptides may provide lead structures for the development of PAR3 agonists or antagonists.

The instant invention is shown and described herein in what is considered to be the most practical, and preferred embodiments. It is recognized, however, that departures may be made therefrom, which are within the scope of the invention, and that obvious modifications will occur to one skilled in the art upon reading this disclosure.

CLAIMS

That which is claimed is:

1. Substantially pure DNA encoding a protease-activated receptor 3.

2. The DNA of claim 1, wherein the DNA is mammalian.

3. Substantially pure DNA having the nucleotide sequence selected from the group consisting of Fig. 1 (SEQ ID NO:1), or degenerate variants thereof, and encoding the amino acid sequence of Fig. 1 (SEQ ID NO:3); Fig. 2 (SEQ ID NO:2), or degenerate variants thereof encoding an amino acid sequence comprising the amino acid sequence of Fig. 1 (SEQ ID NO:3); Fig. 3 (SEQ ID NO:4), or degenerate variants thereof encoding the amino acid sequence of Fig. 2 (SEQ ID NO:6); and Fig. 4 (SEQ ID NO:5), or degenerate variants thereof encoding an amino acid sequence comprising the amino acid sequence of Fig. 3 (SEQ ID NO:6).

4. Substantially pure DNA having 50% or greater sequence identity to the DNA sequence of a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:4 and SEQ ID NO:5 and which hybridizes to the DNA sequence SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, or SEQ ID NO:5, respectively.

5. An isolated protease-activated receptor 3 protein.

6. The substantially pure protein of claim 5 having an amino acid sequence selected from the group consisting of the sequence shown in Fig. 1 (SEQ ID NO:3). and the sequence shown in Fig. 2 (SEQ ID NO:6).

7. A substantially pure polypeptide having an amino acid sequence which is at least 80% identical to an amino acid sequence selected from the group consisting of the sequence shown in Fig. 1 (SEQ ID NO:3) and the sequence shown in Fig. 2 (SEQ ID NO:6), wherein

a) said polypeptide is activated by thrombin; and

b) said polypeptide mediates phosphoinositide hydrolysis in a cell expressing said polypeptide on its surface.

8. A substantially pure polypeptide which is a fragment or analog of a protease-activated receptor 3 comprising a domain capable of activation by thrombin and mediating phosphoinositide hydrolysis.

9. A vector comprising the DNA of claim 1.

10. A cell comprising the vector of claim 9.

11. An assay device, comprising:
a support surface;
and a cell of claim 10.

12. The assay device of claim 11, wherein the cell is bound to the support surface or present in a suspension on the support surface.

13. A method of testing a candidate compound for its ability to act as an agonist of a protease-activated receptor 3 ligand, the method comprising:

a) contacting a candidate compound with a cell which expresses on its surface a recombinant protease-activated receptor 3 protein or biologically active fragment or analog thereof;

b) measuring PAR3-mediated response of the cell; and

c) identifying the candidate compound as an agonist wherein the contacting causes a substantial increase in PAR3-mediated response.

14. A method of testing a candidate compound for the ability to act as an antagonist of a protease-activated receptor 3 ligand, the method comprising:

a) contacting in the presence of a protease-activated receptor agonist a candidate compound with a cell which expresses on its surface a recombinant protease-activated receptor 3 protein or biologically active fragment or analog thereof;

b) measuring PAR3-mediated response of the cell; and

c) identifying the candidate compound as an antagonist wherein the contacting causes a substantial decrease in PAR3-mediated response relative to PAR3-mediated response in the absence of the candidate antagonist.

15. The method of claim 14, wherein the cell is a mammalian cell which normally presents substantially no protease-activated receptor 3 on its surface, the PAR3-mediated response measured in intracellular phosphoinositide hydrolysis in the cell.

16. The method of claim 14, further comprising:

mixing thrombin with platelets and the identified candidate compound; and

observing the effect of the candidate compound on mediating platelet aggregation.

17. A therapeutic composition, comprising:

a protease-activated receptor 3 ligand agonist; and

a physiologically-acceptable carrier.

18. A therapeutic composition, comprising:

a protease-activated receptor 3 ligand antagonist; and

a physiologically-acceptable carrier.

19. The composition of claim 18, wherein the antagonist is selected from the group consisting of:

(1) the isolated sequence LPIKTFRGAPPNSFEEFPFSALE;

(2) uncleavable thrombin inhibitor LPIKPFRGAPPNSFEEFPFSALE where the PAR 3 cleavage site P1' is muted to block cleavage;

(3) uncleavable thrombin inhibitor LP|(hR)TFRGAPPNSFEEFPFSALE where the PAR 3 cleavage site P1 is mutated to block cleavage;
hR is beta-homoarginine (the extra methylene group is in the main chain);

(4) uncleavable thrombin inhibitor (dF)PRPFRGAPPNSFEEFPFSALE where the good active site binding sequence dFPR is substituted for LPIK; dF is D-Phenylalanine;

(5) any of (1)-(4) above where all or part of the sequence TFRGAPPNS is replaced with spacer sequences such as GGG;

(6) variations and combinations of (1)-(5) which act as antagonists.

20. A method of treatment, comprising:
administering to a patient a therapeutically effective amount of the composition of claim 18.

Attorney Docket No.: 06510/060001

PROTEASE-ACTIVATED RECEPTOR 3 AND USES THEREOF

Abstract of the Disclosure

Disclosed are cDNAs and genomic DNAs encoding protease-activated receptor 3 (PAR3) from mouse and human, and the recombinant polypeptides expressed from such cDNAs. The recombinant receptor polypeptides, receptor fragments and analogs expressed on the surface of cells are used in methods of screening candidate compounds for their ability to act as agonists or antagonists to the effects of interaction between thrombin and PAR3. Agonists are used as therapeutics to treat wounds, thrombosis, atherosclerosis, restenosis, inflammation, and other thrombin-activated disorders. Antagonists are used as therapeutics to control blood coagulation and thereby treating heart attack and stroke. Antagonists mediate inflammatory and proliferative responses to injury as occur in normal wound healing and variety of diseases including atherosclerosis, restenosis, pulmonary inflammation (ARDS) and glomerulosclerosis. Antibodies specific for a protease-activated receptor 3 (or receptor fragment or analog) and their use as a therapeutic are also disclosed.

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Atty Docket No. 06510/060DIV

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of:

Shaun R. Coughlin et al.

Serial No.: Unassigned (Divisional of USSN 08/742,440) Group Art Unit: Unassigned

Filing Date: December 8, 1998 Examiner: Unassigned

Title: PROTEASE-ACTIVATED RECEPTOR 3 AND USES THEREOF

SUBMISSION OF FORMAL DRAWINGS

Assistant Commissioner for Patents
Washington, D.C. 20231
Attention: Official Draftsman

Sir:

Enclosed are 16 sheets of formal drawings in connection with the above identified case.

The Commissioner is hereby authorized to charge any fees under 37 C.F.R. §§ 1.16 and 1.17 which may be required by this paper, or to credit any overpayment, to Deposit Account No. 18-0580.

Respectfully submitted,

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FIG. 1-1

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               10          20           30          40          50
     *     *     *    *     *     *     *     *     *     *
 TG ACT TTG TAT ACT TAA CAA CAT CCT GTA GCC GGG TCT CAG GAC ATC AAG
 AC TGA AAC ATA TGA ATT GTT GTA GGA CAT CGG CCC AGA GTC CTG TAG TTC
    T   L   Y   T   *   Q   H   P   V   A   G   S   Q   D   I   K>
               60          70          80          90
     *     *     *    *     *     *     *     *     *
 ATG AAA ATC CTT ATC TTG GTT GCA GCT GGG CTG CTG TTT CTG CCA GTC
 TAC TTT TAG GAA TAG AAC CAA CGT CGA CCC GAC GAC AAA GAC GGT CAG
  M   K   I   L   I   L   V   A   A   G   L   L   F   L   P   V>
100         110         120         130         140
  *     *     *     *     *     *     *     *     *     *
 ACT GTT TGC CAA AGT GGC ATA AAT GTT TCA GAC AAC TCA GCA AAG CCA
 TGA CAA ACG GTT TCA CCG TAT TTA CAA AGT CTG TTG AGT CGT TTC GGT
  T   V   C   Q   S   G   I   N   V   S   D   N   S   A   K   P>
   150         160         170          180         190
     *     *     *     *     *     *     *     *     *
 ACC TTA ACT ATT AAG AGT TTT AAT GGG GGT CCC CAA AAT ACC TTT GAA
 TGG AAT TGA TAA TTC TCA AAA TTA CCC CCA GGG GTT TTA TGG AAA CTT
  T   L   T   I   K   S   F   N   G   G   P   Q   N   T   F   E>
     200         210         220         230          240
 *     *     *     *     *     *     *     *     *     *
 GAA TTC CCA CTT TCT GAC ATA GAG GGC TGG ACA GGA GCC ACC ACA ACT
 CTT AAG GGT GAA AGA CTG TAT CTC CCG ACC TGT CCT CGG TGG TGT TGA
  E   F   P   L   S   D   I   E   G   W   T   G   A   T   T   T>
        250         260          270         280         290
   *     *     *     *     *     *     *     *     *     *
 ATA AAA GCG GAG TGT CCC GAG GAC AGT ATT TCA ACT CTC CAC GTG AAT
 TAT TTT CGC CTC ACA GGG CTC CTG TCA TAA AGT TGA GAG GTG CAC TTA
  I   K   A   E   C   P   E   D   S   I   S   T   L   H   V   N>
              300         310         320          330
     *     *     *    *     *     *     *     *     *
 AAT GCT ACC ATA GGA TAC CTG AGA AGT TCC TTA AGT ACC CAA GTG ATA
 TTA CGA TGG TAT CCT ATG GAC TCT TCA AGG AAT TCA TGG GTT CAC TAT
  N   A   T   I   G   Y   L   R   S   S   L   S   T   Q   V   I>
340         350         360         370         380
  *     *     *     *     *     *     *     *     *     *
 CCT GCC ATC TAT ATC CTG CTG TTT GTG GTT GGT GTA CCA TCC AAC ATC
 GGA CGG TAG ATA TAG GAC GAC AAA CAC CAA CCA CAT GGT AGG TTG TAG
  P   A   I   Y   I   L   L   F   V   V   G   V   P   S   N   I>
   390         400         410          420         430
     *     *     *     *     *     *     *     *     *
 GTG ACC CTG TGG AAA CTC TCC TTA AGG ACC AAA TCC ATC AGT CTG GTC
 CAC TGG GAC ACC TTT GAG AGG AAT TCC TGG TTT AGG TAG TCA GAC CAG
  V   T   L   W   K   L   S   L   R   T   K   S   I   S   L   V>
```

FIG. 1-2

```
     440          450          460          470          480
 *     *     *     *     *     *     *     *     *     *
ATC TTT CAC ACC AAC CTG GCC ATC GCA GAT CTC CTT TTC TGT GTC ACA
TAG AAA GTG TGG TTG GAC CGG TAG CGT CTA GAG GAA AAG ACA CAG TGT
 I   F   H   T   M   L   A   I   A   D   L   L   F   C   V   T>
        490          500          510          520          530
  *     *     *     *     *     *     *     *     *     *
CTG CCA TTT AAG ATC GCC TAC CAT CTC AAT GGC AAC AAC TGG GTA TTT
GAC GGT AAA TTC TAG CGG ATG GTA GAG TTA CCG TTG TTG ACC CAT AAA
 L   P   F   K   I   A   Y   H   L   N   G   N   N   W   V   F>
           540          550          560          570
     *     *     *     *     *     *     *     *     *
GGC GAG GTC ATG TGC CGG ATC ACC ACG GTC GTT TTC TAC GGC AAC ATG
CCG CTC CAG TAC ACG GCC TAG TGG TGC CAG CAA AAG ATG CCG TTG TAC
 G   E   V   M   C   R   I   T   T   V   V   F   Y   G   N   M>
580          590          600          610          620
 *     *     *     *     *     *     *     *     *     *
TAC TGC GCT ATC CTG ATC CTC ACT TGC ATG GGC ATC AAC CGC TAC CTG
ATG ACG CGA TAG GAC TAG GAG TGA ACG TAC CCG TAG TTG GCG ATG GAC
 Y   C   A   I   L   I   L   T   C   M   G   I   N   R   Y   L>
  630          640          650          660          670
     *     *     *     *     *     *     *     *     *
GCC ACG GCT CAC CCT TTC ACA TAC CAG AAG CTG CCC AAA CGC AGC TTC
CGG TGC CGA GTG GGA AAG TGT ATG GTC TTC GAC GGG TTT GCG TCG AAG
 A   T   A   H   P   F   T   Y   Q   K   L   P   K   R   S   F>
     680          690          700          710          720
 *     *     *     *     *     *     *     *     *     *
TCC TTG CTC ATG TGT GGC ATA GTG TGG GTC ATG GTT TTC TTA TAC ATG
AGG AAC GAG TAC ACA CCG TAT CAC ACC CAG TAC CAA AAG AAT ATG TAC
 S   L   L   M   C   G   I   V   W   V   M   V   F   L   Y   M>
        730          740          750          760          770
  *     *     *     *     *     *     *     *     *     *
CTG CCC TTT GTC ATC CTG AAG CAG GAG TAC CAC CTC GTC CAC TCA GAG
GAC GGG AAA CAG TAG GAC TTC GTC CTC ATG GTG GAG CAG GTG AGT CTC
 L   P   F   V   I   L   K   Q   E   Y   H   L   V   H   S   E>
           780          790          800          810
     *     *     *     *     *     *     *     *     *
ATC ACC ACC TGC CAC GAT GTC GTC GAC GCG TGC GAG TCC CCA TCA TCC
TAG TGG TGG ACG GTG CTA CAG CAG CTG CGC ACG CTC AGG GGT AGT AGG
 I   T   T   C   H   D   V   V   D   A   C   E   S   P   S   S>
820          830          840          850          860
 *     *     *     *     *     *     *     *     *     *
TTC CGA TTC TAC TAC TTC GTC TCC TTA GCA TTC TTT GGG TTC CTC ATC
AAG GCT AAG ATG ATG AAG CAG AGG AAT CGT AAG AAA CCC AAG GAG TAG
 F   R   F   Y   Y   F   V   S   L   A   F   F   G   F   L   I>
```

FIG. 1-3

```
  870         880         890          900         910
    *    *     *     *     *     *     *     *     *
CCG TTT GTG ATC ATC ATC TTC TGT TAC ACG ACT CTC ATC CAC AAA CTT
GGC AAA CAC TAG TAG TAG AAG ACA ATG TGC TGA GAG TAG GTG TTT GAA
 P   F   V   I   I   I   F   C   Y   T   T   L   I   H   K   L>
    920         930         940         950         960
 *    *     *     *     *     *     *     *     *     *
AAA TCA AAG GAT CGG ATA TGG CTG GGC TAC ATC AAG GCC GTC CTC CTC
TTT AGT TTC CTA GCC TAT ACC GAC CCG ATG TAG TTC CGG CAG GAG GAG
 K   S   K   D   R   I   W   L   G   Y   I   K   A   V   L   L>
       970         980         990         1000        1010
  *     *     *     *     *     *     *     *     *     *
ATC CTT GTG ATT TTC ACA ATT TGC TTT GCC CCC ACC AAC ATC ATA CTC
TAG GAA CAC TAA AAG TGT TAA ACG AAA CGG GGG TGG TTG TAG TAT GAG
 I   L   V   I   F   T   I   C   F   A   P   T   N   I   I   L>
         1020        1030        1040        1050
     *     *     *     *     *     *     *     *     *
GTA ATC CAC CAT GCC AAC TAC TAC TAC CAC AAT ACC GAC AGC TTG TAC
CAT TAG GTG GTA CGG TTG ATG ATG ATG GTG TTA TGG CTG TCG AAC ATG
 V   I   H   H   A   N   Y   Y   Y   H   N   T   D   S   L   Y>
1060        1070        1080        1090        1100
  *     *     *     *     *     *     *     *     *     *
TTT ATG TAT CTT ATT GCT CTG TGC CTG GGG AGC CTG AAT AGC TGC CTA
AAA TAC ATA GAA TAA CGA GAC ACG GAC CCC TCG GAC TTA TCG ACG GAT
 F   M   Y   L   I   A   L   C   L   G   S   L   N   S   C   L>
 1100        1120        1130        1140        1150
    *    *     *     *     *     *     *     *     *
GAT CCA TTC CTT TAC TTT GTC ATG TCG AAA GTT GTA GAT CAG CTT AAT
CTA GGT AAG GAA ATG AAA CAG TAC AGC TTT CAA CAT CTA GTC GAA TTA
 D   P   F   L   Y   F   V   M   S   K   V   V   D   Q   L   N>
   1160        1170        1180        1190        1200
 *    *     *     *     *     *     *     *     *     *
CCT TAG TCG GCA ATG GCA AGA CCA CTT TAG AGA CCA AGG AGA GAT ATC
GGA ATC AGC CGT TAC CGT TCT GGT GAA ATC TCT GGT TCC TCT CTA TAG
 P   *   S   A   M   A   R   P   L   *   R   P   R   R   D   I>
      1210        1220
  *     *     *     *
TGG GAA GAC ATA CAT GCT TGG C
ACC CTT CTG TAT GTA CGA ACC G
 W   E   D   I   H   A   W   X>
```

FIG. 2-1

```
        10         20         30         40         50
    *    *     *    *     *    *     *    *     *    *
CCATATGCTA ATATTTCCTT TCAATTACAG GCATAAATGT TTCAGACAAC

        60         70         80         90        100
    *    *     *    *     *    *     *    *     *    *
TCAGCAAAGC CAACCTTAAC TATTAAGAGT TTTAATGGGG GTCCCCAAAA

       110        120        130        140        150
    *    *     *    *     *    *     *    *     *    *
TACCTTTGAA GAATTC---- ---TACAACT CTCCATGTGA ATAATGCTAC

       160        170        180        190        200
    *    *     *    *     *    *     *    *     *    *
CATGGGATAC CTGAGAAGTT CCTTAAGTAC CAAAGTGATA CCTGCCATCT

       210        220        230        240        250
    *    *     *    *     *    *     *    *     *    *
ACATCCTGGT GTTTGTGATT GGTGTACCAG CGAACATCGT GACCCTGTGG

       260        270        280        290        300
    *    *     *    *     *    *     *    *     *    *
AAACTCTCCT CAAGGACCAA ATCCATCTGT CTGGTCATCT TTCACACCAA

       310        320        330        340        350
    *    *     *    *     *    *     *    *     *    *
CCTGGCCATC GCGGATCTCC TTTTCTGTGT CACGCTGCCG TTTAAGATC-

       360        370        380        390        400
    *    *     *    *     *    *     *    *     *    *
-CCTACCATC TCAATGGCAA CAACTGGGTA TTTGGCGAGG TCATGTGCCG

       410        420        430        440        450
    *    *     *    *     *    *     *    *     *    *
GATCACCACG GTCGTTTTCT ACGGCAACAT GTACTGCGCT A---TCCTGA

       460        470        480        490        500
    *    *     *    *     *    *     *    *     *    *
TCCTCACCTG CATGGGCATC AACCGCTACC TGGCCACGGC TCACCCTTTC

       510        520        530        540        550
    *    *     *    *     *    *     *    *     *    *
ACATACCAGA AGCTGCCCAA ACGCAGCTTC TCCATGCTCA TGTGTGGCAT

       560        570        580        590        600
    *    *     *    *     *    *     *    *     *    *
GGTGTGGGTC ATGGTTTTCT TATACATGCT GCCCTTTGTC ATCC---AAG

       610        620        630        640        650
    *    *     *    *     *    *     *    *     *    *
CAGGAGTACC ACCTCGTCCA CTCCGAGATC ACCACCTGCC ACGATGTCGT
```

FIG. 2-2

```
       660        670        680        690        700
   *    *     *    *     *    *     *    *     *    *
CGACGCGTGC GANTCCCCAT CATCCTTCCG ATTCTACTAC TTCGTCTCCT
       710        720        730        740        750
   *    *     *    *     *    *     *    *     *    *
TAGCATTCTT TGGGTTCCTC ATCCCGTTTG TGATCATCAT CTTCTGTTAC
       760        770        780        790        800
   *    *     *    *     *    *     *    *     *    *
ACGACTCTCA TCCACAAACT TAAATCAAAA GATCNGATAT GGCTGGGCTA
       810        820        830        840        850
   *    *     *    *     *    *     *    *     *    *
CATCAAGGCC GTCCTCCTCA TCCTTGTGAA TTTCACCATC TGCTTCCCCC
       860        870        880        890        900
   *    *     *    *     *    *     *    *     *    *
CCACCAAG-- ----GATATC TGGGAAGACG TACATGCTTG GCTGACTTGT
       910        920        930        940        950
   *    *     *    *     *    *     *    *     *    *
GCATGGCACC ATCAGCTCAA TTTTTAATTT TTTAATTTTA ATTTAATTTA
       960        970        980        990       1000
   *    *     *    *     *    *     *    *     *    *
ATTTTATGTT TTTGAGACAG AGCCTCACTG TGTAGTCCTG GCTGGCCTGG
      1010       1020       1030       1040       1050
   *    *     *    *     *    *     *    *     *    *
CTGGTTCTCT ATTTAGACCA GGTTAGCCTT GAACTCACAG AGATCTGCCT
      1060       1070       1080       1090       1100
   *    *     *    *     *    *     *    *     *    *
GCTTCTGCCT CCCAAGTGCT GGGTTCAACC AGGTCTGGCA AGCGCTCCAT
      1110       1120
   *    *     *    *
TTTTCAGCTC CTCTGCAACA GTGC
```

FIG. 3-1

```
               10              20              30              40
       *       *       *       *       *       *       *       *       *
TGC TCC ATG ATT TTA CAG ATT TCA TAA CGT TTA AGA GAC GGG ACT CAG
ACG AGG TAC TAA AAT GTC TAA AGT ATT GCA AAT TCT CTG CCC TGA GTC
 C   S   M   I   L   Q   I   S   *   R   L   R   D   G   T   Q>
50              60              70              80              90
 *       *       *       *       *       *       *       *       *       *
GTC ATC AAA ATG AAA GCC CTC ATC TTT GCA GCT GCT GGC CTC CTG CTT
CAG TAG TTT TAC TTT CGG GAG TAG AAA CGT CGA CGA CCG GAG GAC GAA
 V   I   K   M   K   A   L   I   F   A   A   A   G   L   L   L>
  100             110             120             130             140
     *       *       *       *       *       *       *       *       *
CTG TTG CCC ACT TTT TGT CAG AGT GGC ATG GAA AAT GAT ACA AAC AAC
GAC AAC GGG TGA AAA ACA GTC TCA CCG TAC CTT TTA CTA TGT TTG TTG
 L   L   P   T   F   C   Q   S   G   M   E   N   D   T   N   N>
     150             160             170             180             190
 *       *       *       *       *       *       *       *       *       *
TTG GCA AAG CCA ACC TTA CCC ATT AAG ACC TTT CGT GGA GCT CCC CCA
AAC CGT TTC GGT TGG AAT GGG TAA TTC TGG AAA GCA CCT CGA GGG GGT
 L   A   K   P   T   L   P   I   K   T   F   R   G   A   P   P>
         200             210             220             230             240
   *       *       *       *       *       *       *       *       *       *
AAT TCT TTT GAA GAG TTC CCC TTT TCT GCC TTG GAA GGC TGG ACA GGA
TTA AGA AAA CTT CTC AAG GGG AAA AGA CGG AAC CTT CCG ACC TGT CCT
 N   S   F   E   E   F   P   F   S   A   L   E   G   W   T   G>
             250             260             270             280
       *       *       *       *       *       *       *       *       *
GCC ACG ATT ACT GTA AAA ATT AAG TGC CCT GAA GAA AGT GCT TCA CAT
CGG TGC TAA TGA CAT TTT TAA TTC ACG GGA CTT CTT TCA CGA AGT GTA
 A   T   I   T   V   K   I   K   C   P   E   E   S   A   S   H>
290             300             310             320             330
 *       *       *       *       *       *       *       *       *       *
CTC CAT GTG AAA AAT GCT ACC ATG GGG TAC CTG ACC AGC TCC TTA AGT
GAG GTA CAC TTT TTA CGA TGG TAC CCC ATG GAC TGG TCG AGG AAT TCA
 L   H   V   K   N   A   T   M   G   Y   L   T   S   S   L   S>
  340             350             360             370             380
     *       *       *       *       *       *       *       *       *
ACT AAA CTG ATA CCT GCC ATC TAC CTC CTG GTG TTT GTA GTT GGT GTC
TGA TTT GAC TAT GGA CGG TAG ATG GAG GAC CAC AAA CAT CAA CCA CAG
 T   K   L   I   P   A   I   Y   L   L   V   F   V   V   G   V>
     390             400             410             420             430
 *       *       *       *       *       *       *       *       *       *
CCG GCC AAT GCT GTG ACC CTG TGG ATG CTT TTC TTC AGG ACC AGA TCC
GGC CGG TTA CGA CAC TGG GAC ACC TAC GAA AAG AAG TCC TGG TCT AGG
 P   A   N   A   V   T   L   W   M   L   F   F   R   T   R   S>
```

FIG. 3-2

```
        440        450        460        470        480
   *     *     *     *     *     *     *     *     *     *
ATC TGT ACC ACT GTA TTC TAC ACC AAC CTG GCC ATT GCA GAT TTT CTT
TAG ACA TGG TGA CAT AAG ATG TGG TTG GAC CGG TAA CGT CTA AAA GAA
 I   C   T   T   V   F   Y   T   N   L   A   I   A   D   F   L>
           490        500        510        520
     *     *     *     *     *     *     *     *     *
TTT TGT GTT ACA TTG CCC TTT AAG ATA GCT TAT CAT CTC AAT GGG AAC
AAA ACA CAA TGT AAC GGG AAA TTC TAT CGA ATA GTA GAG TTA CCC TTG
 F   C   V   T   L   P   F   K   I   A   Y   H   L   N   G   N>
530        540        550        560        570
  *     *     *     *     *     *     *     *     *     *
AAC TGG GTA TTT GGA GAG GTC CTG TGC CGG GCC ACC ACA GTC ATC TTC
TTG ACC CAT AAA CCT CTC CAG GAC ACG GCC CGG TGG TGT CAG TAG AAG
 N   W   V   F   G   E   V   L   C   R   A   T   T   V   I   F>
   580        590        600        610        620
     *     *     *     *     *     *     *     *     *
TAT GGC AAC ATG TAC TGC TCC ATT CTG CTC CTT GCC TGC ATC AGC ATC
ATA CCG TTG TAC ATG ACG AGG TAA GAC GAG GAA CGG ACG TAG TCG TAG
 Y   G   N   M   Y   C   S   I   L   L   L   A   C   I   S   I>
     630        640        650        660        670
 *     *     *     *     *     *     *     *     *     *
AAC CGC TAC CTG GCC ATC GTC CAT CCT TTC ACC TAC CGG GGC CTG CCC
TTG GCG ATG GAC CGG TAG CAG GTA GGA AAG TGG ATG GCC CCG GAC GGG
 N   R   Y   L   A   I   V   H   P   F   T   Y   R   G   L   P>
        680        690        700        710        720
   *     *     *     *     *     *     *     *     *     *
AAG CAC ACC TAT GCC TTG GTA ACA TGT GGA CTG GTG TGG GCA ACA GTT
TTC GTG TGG ATA CGG AAC CAT TGT ACA CCT GAC CAC ACC CGT TGT CAA
 K   H   T   Y   A   L   V   T   C   G   L   V   W   A   T   V>
           730        740        750        760
     *     *     *     *     *     *     *     *     *
TTC TTA TAT ATG CTG CCA TTT TTC ATA CTG AAG CAG GAA TAT TAT CTT
AAG AAT ATA TAC GAC GGT AAA AAG TAT GAC TTC GTC CTT ATA ATA GAA
 F   L   Y   M   L   P   F   F   I   L   K   Q   E   Y   Y   L>
770        780        790        800        810
  *     *     *     *     *     *     *     *     *     *
GTT CAG CCA GAC ATC ACC ACC TGC CAT GAT GTT CAC AAC ACT TGC GAG
CAA GTC GGT CTG TAG TGG TGG ACG GTA CTA CAA GTG TTG TGA ACG CTC
 V   Q   P   D   I   T   T   C   H   D   V   H   N   T   C   E>
   820        830        840        850        860
     *     *     *     *     *     *     *     *     *
TCC TCA TCT CCC TTC CAA CTC TAT TAC TTC ATC TCC TTG GCA TTC TTT
AGG AGT AGA GGG AAG GTT GAG ATA ATG AAG TAG AGG AAC CGT AAG AAA
 S   S   S   P   F   Q   L   Y   Y   F   I   S   L   A   F   F>
```

FIG. 3-3

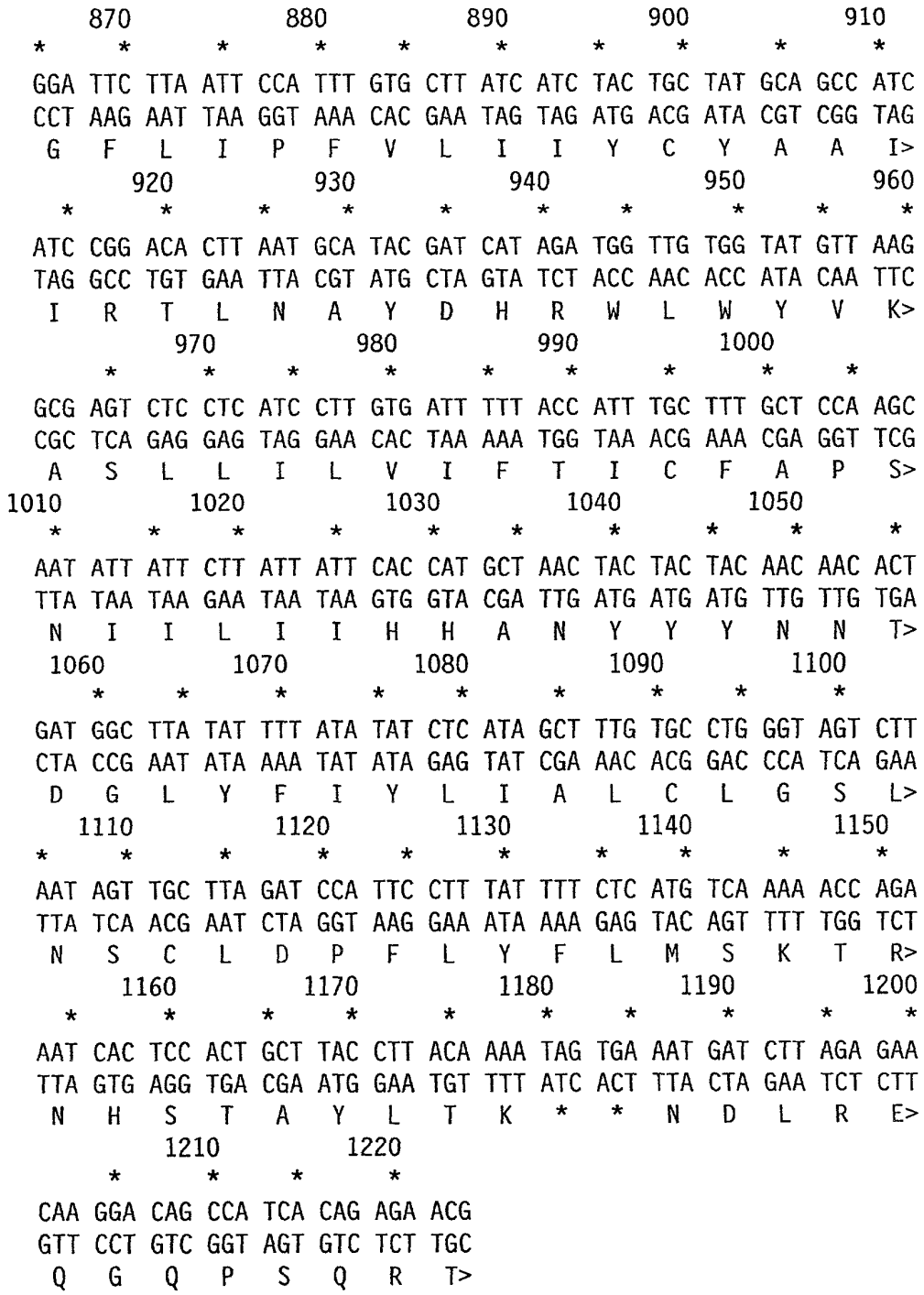

870 880 890 900 910
GGA TTC TTA ATT CCA TTT GTG CTT ATC ATC TAC TGC TAT GCA GCC ATC
CCT AAG AAT TAA GGT AAA CAC GAA TAG TAG ATG ACG ATA CGT CGG TAG
G F L I P F V L I I Y C Y A A I>
920 930 940 950 960
ATC CGG ACA CTT AAT GCA TAC GAT CAT AGA TGG TTG TGG TAT GTT AAG
TAG GCC TGT GAA TTA CGT ATG CTA GTA TCT ACC AAC ACC ATA CAA TTC
I R T L N A Y D H R W L W Y V K>
970 980 990 1000
GCG AGT CTC CTC ATC CTT GTG ATT TTT ACC ATT TGC TTT GCT CCA AGC
CGC TCA GAG GAG TAG GAA CAC TAA AAA TGG TAA ACG AAA CGA GGT TCG
A S L L I L V I F T I C F A P S>
1010 1020 1030 1040 1050
AAT ATT ATT CTT ATT ATT CAC CAT GCT AAC TAC TAC TAC AAC AAC ACT
TTA TAA TAA GAA TAA TAA GTG GTA CGA TTG ATG ATG ATG TTG TTG TGA
N I I L I I H H A N Y Y Y N N T>
1060 1070 1080 1090 1100
GAT GGC TTA TAT TTT ATA TAT CTC ATA GCT TTG TGC CTG GGT AGT CTT
CTA CCG AAT ATA AAA TAT ATA GAG TAT CGA AAC ACG GAC CCA TCA GAA
D G L Y F I Y L I A L C L G S L>
1110 1120 1130 1140 1150
AAT AGT TGC TTA GAT CCA TTC CTT TAT TTT CTC ATG TCA AAA ACC AGA
TTA TCA ACG AAT CTA GGT AAG GAA ATA AAA GAG TAC AGT TTT TGG TCT
N S C L D P F L Y F L M S K T R>
1160 1170 1180 1190 1200
AAT CAC TCC ACT GCT TAC CTT ACA AAA TAG TGA AAT GAT CTT AGA GAA
TTA GTG AGG TGA CGA ATG GAA TGT TTT ATC ACT TTA CTA GAA TCT CTT
N H S T A Y L T K * * N D L R E>
1210 1220
CAA GGA CAG CCA TCA CAG AGA ACG
GTT CCT GTC GGT AGT GTC TCT TGC
Q G Q P S Q R T>

FIG. 4-1

```
       10         20         30         40         50
   *   *   *   *   *   *   *   *   *   *
-ACAGGCATG GAAAATGATA CAAACAACTT GGCAAAGCCA ACCTTACCCA

       60         70         80         90        100
   *   *   *   *   *   *   *   *   *   *
TTAAGACCTT TCGTGGAGCT CCCCCAAATT CTTTTGAAGA GTTCCCCTTT

      110        120        130        140        150
   *   *   *   *   *   *   *   *   *   *
TCTGCCTTGG AAGGCTGGAC AGGAGCCACG ATTACTGTAA AAATTAAGTG

      160        170        180        190        200
   *   *   *   *   *   *   *   *   *   *
CCCTGAAGAA AGTGCTTCAC ATCTCCATGT GAAAAATGCT ACCATGGGGT

      210        220        230        240        250
   *   *   *   *   *   *   *   *   *   *
ACCTGACCAG CTCCTTAAGT ACTAAACTGA TACCTGCCAT CTACCTCCTG

      260        270        280        290        300
   *   *   *   *   *   *   *   *   *   *
GTGTTTGTAG TTGGTGTCCC GGCCAATGCT GTGACCCTGT GGATGCTTTT

      310        320        330        340        350
   *   *   *   *   *   *   *   *   *   *
CTTCAGGACC AGATCCATCT GTACCACTGT ATTCTACACC AACCTGGCCA

      360        370        380        390        400
   *   *   *   *   *   *   *   *   *   *
TTGCAGATTT TCTTTTTTGT GTTACATTGC CCTTTAAGAT AGCTTATCAT

      410        420        430        440        450
   *   *   *   *   *   *   *   *   *   *
CTCAATGGGA ACAACTGGGT ATTTGGAGAG GTCCTGTGCC GGGCCACCAC

      460        470        480        490        500
   *   *   *   *   *   *   *   *   *   *
AGTCATCTTC TATGGCAACA TGTACTGCTC CATTCTGCTC CTTGCCTGCA

      510        520        530        540        550
   *   *   *   *   *   *   *   *   *   *
TCAGCATCAA CCGCTACCTG GCCATCGTCC ATCCTTTCAC CTACCGGGGC

      560        570        580        590        600
   *   *   *   *   *   *   *   *   *   *
CTGCCCAAGC ACACCTATGC CTTGGTAACA TGTGGACTGG TGTGGGCAAC

      610        620        630        640        650
   *   *   *   *   *   *   *   *   *   *
AGTTTTCTTA TATATGCTGC CATTTTTCAT ACTGAAGCAG GAATATTATC
```

FIG. 4-2

```
      660        670        680        690        700
    *    *     *    *     *    *     *    *     *    *
TTGTTCAGCC AGACATCACC ACCTGCCATG ATGTTCACAA CACTTGCGAG

      710        720        730        740        750
    *    *     *    *     *    *     *    *     *    *
TCCTCATCTC CCTTCCAACT CTATTACTTC ATCTCCTTGG CATTCTTTGG

      760        770        780        790        800
    *    *     *    *     *    *     *    *     *    *
ATTCTTAATT CCATTTGTGC TTATCATCTA CTGCTATGCA GCCATCATCC

      810        820        830        840        850
    *    *     *    *     *    *     *    *     *    *
GGACACTTAA TGCATACGAT CATAGATGGT TGTGGTATGT TAAGGCGAGT

      860        870        880        890        900
    *    *     *    *     *    *     *    *     *    *
CTCCTCATCC TTGTGATTTT TACCATTTGC TTTGCTCCAA GCAATATTAT

      910        920        930        940        950
    *    *     *    *     *    *     *    *     *    *
TCTTATTATT CACCATGCTA ACTACTACTA CAACAACACT GATGGCTTAT

      960        970        980        990       1000
    *    *     *    *     *    *     *    *     *    *
ATTTTATATA TCTCATAGCT TTGTGCCTGG GTAGTCTTAA TAGTTGCTTA

     1010       1020       1030       1040       1050
    *    *     *    *     *    *     *    *     *    *
GATCCATTCC TTTATTTTCT CATGTCAAAA ACCAGAAATC ACTCCACTGC

     1060       1070       1080       1090       1100
    *    *     *    *     *    *     *    *     *    *
TTACCTTACA AAATAGTGAA ATGATCTTAG AGAACAAGGA CAGCCATCAC

AGA
```

FIG. 5A

```
hPAR3-1 MKA   LIFAAAGLLLLLP TFCQSGMENDTNNLAKP TLPIK/TFRGAPPN SFEEFPFSALEGWTGATITVKIKC     PEESASHLHVKNATMG
hPAR1-1 MGPRR LLLVAACFSLCGP LLSARTRARRPESKATNATLDPR/SFLLRNPNDKYEPFWEDEEKNESGLTEYRLVSINKSSPLQKQLPAFISEDASG
hPAR2-1 MRSPSAAWLLGAAILLA ASLSCSGTIQG      TNRSSKGR/SLIGKVDGTSHVTGKGVTV                                ETVFSVDEFSAS

                 |---------TM1---------|         |----------TM2---------|                          |---------TM3----------|
hPAR3-87 YLTSSLSTKLIPAIYLLVFVVGVPANAVTLWMLFFRTR SICTTVFYTNLAIADFLFCVTLPFKIAYHLNGNNWVFGEVLCRATTVIFYGNMYCSILLLACISINRYLAI
hPAR1-95 YLTSSWLTLFVPSVYTGVFVVSLPLNIMAIVVFILKMKVKKPAVVYMLHLATADVLFVSVLPFKISYYFSGSDWQFGSELCRFVTAAFYCNMYASILLMTVISIDRFLAV
hPAR2-68 VLTGKLTTVFLPIVYTIVFVVGLPSNGMALWVFLFRTKKKHPAVIYMANLALADLLSVIWFPLKIAYHIHGNNWIYGEALCNVLIGFFYGNMYCSILFMTCLSVQRYWVI

                    |----------TM4----------|                                          |----------TM5-----------|
hPAR3-196 VHPFTYRGLPKHTYALVTCGLVWATVFLYMLPFFILKQEYYLVQPDITTCHDVHNTCESSSPFQLYYFISLAFFGFLIPFVLIIYCYAAIIRTLNA      YDHRWLWYV
hPAR1-205 VYPMQSLSWRTLGRASFTCLAIWALAIAGVVPLVLKEQTIQVPGLNITTCHDVLNETLLEG YYAYYFSAFSAVFFFVPLIISTVCYCSIIRCLSSSAVANRSKK  SRAL
hPAR2-178 VNPMGHSRKKANIAIGI SLAIWLLILLVTIPLYVVKQTIFIPALNITTCHDVLPEQLLVGD MFNYFLSLAIGVFLFPAFLTASAYVLMIRMLRSSAMDENSEKKRKRAI

          |----------TM6--------|                 |-------------TM7--------|
hPAR3-301 KASLLILVIFTICFAPSNIILIIHHANYYYNNT DGLYFIYLIALCLGSLNSCLDPFLYFLMSKTRNHSTAYLTK
hPAR1-313 FLSAAVFCIFIICFGPTNVLLIAHYSFLSHTSTTEAAYFAYLLCVCVSSISSCIDPLIYYYASSECQRYVYSILCCKESSDPSSYNSSGQLMASKMDTCSSNLNNSIYKFLLT
hPAR2-287 KLIVTVLAMYLICFTPSNLLLVVHY FLIKSQGQSHVYALYIVALCLSTLNSCIDPFVYYFVSHDFRDHAKNALLCRSVRTVKQMQVSLTSKKHSRKSSSYSSSSTTVKTSY
```

FIG. 5B

```
Hirudin C-tail                  ..DFEEIPEEYLQ
hPAR3- 34-62 ..TLPIK / TFRGAPPN SFEEFPFSALEGWTGA..
hPAR1- 37-65 ..TLDPR / SFLLRNPNDKYEPFWEDEEKNESG..
hPAR2- 32-62 ..SSKGR / SLIGKVDGTSHVTGKGVTVETVFSVD..
```

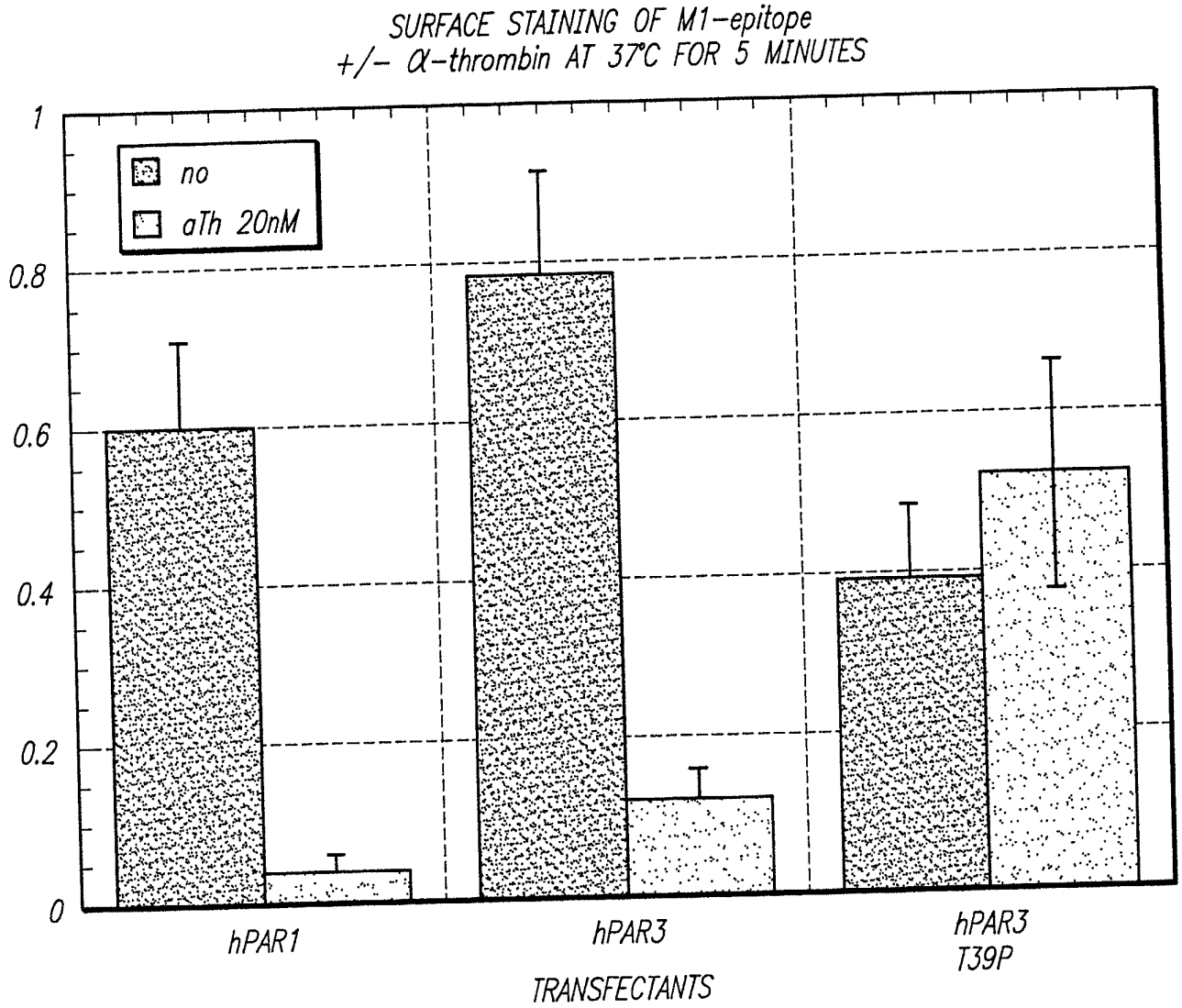

SURFACE STAINING OF M1-epitope
+/- α-thrombin AT 37°C FOR 5 MINUTES
1
0.8
0.6
0.4
0.2
0
no
aTh 20nM
hPAR1
hPAR3
hPAR3
T39P
TRANSFECTANTS

FIG. 6

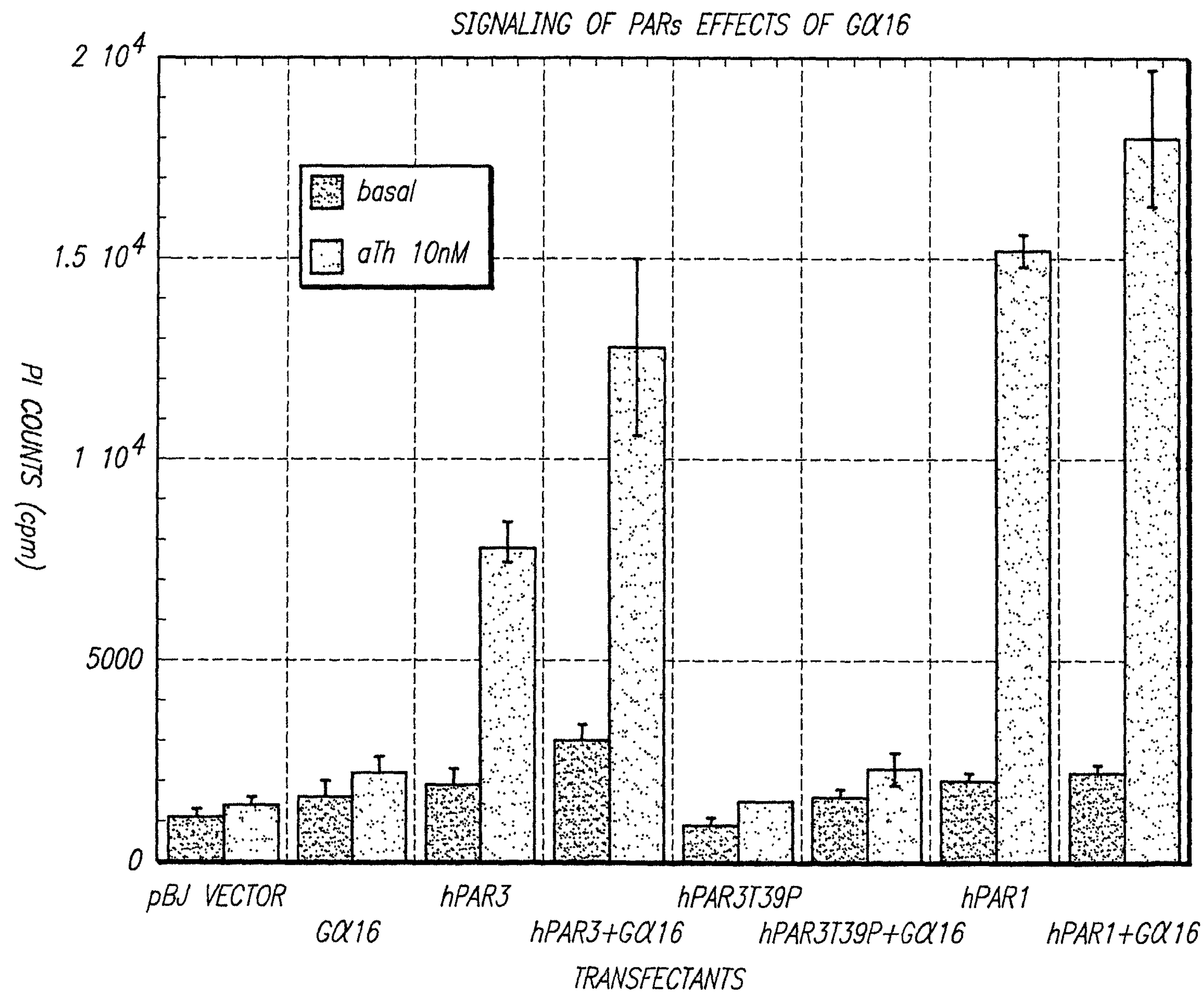

SIGNALING OF PARs EFFECTS OF Gα16
basal
aTh 10nM
2 10^4
1.5 10^4
1 10^4
5000
0
PI COUNTS (cpm)
pBJ VECTOR
Gα16
hPAR3
hPAR3+Gα16
hPAR3T39P
hPAR3T39P+Gα16
hPAR1
hPAR1+Gα16
TRANSFECTANTS

FIG. 7

FIG. 8

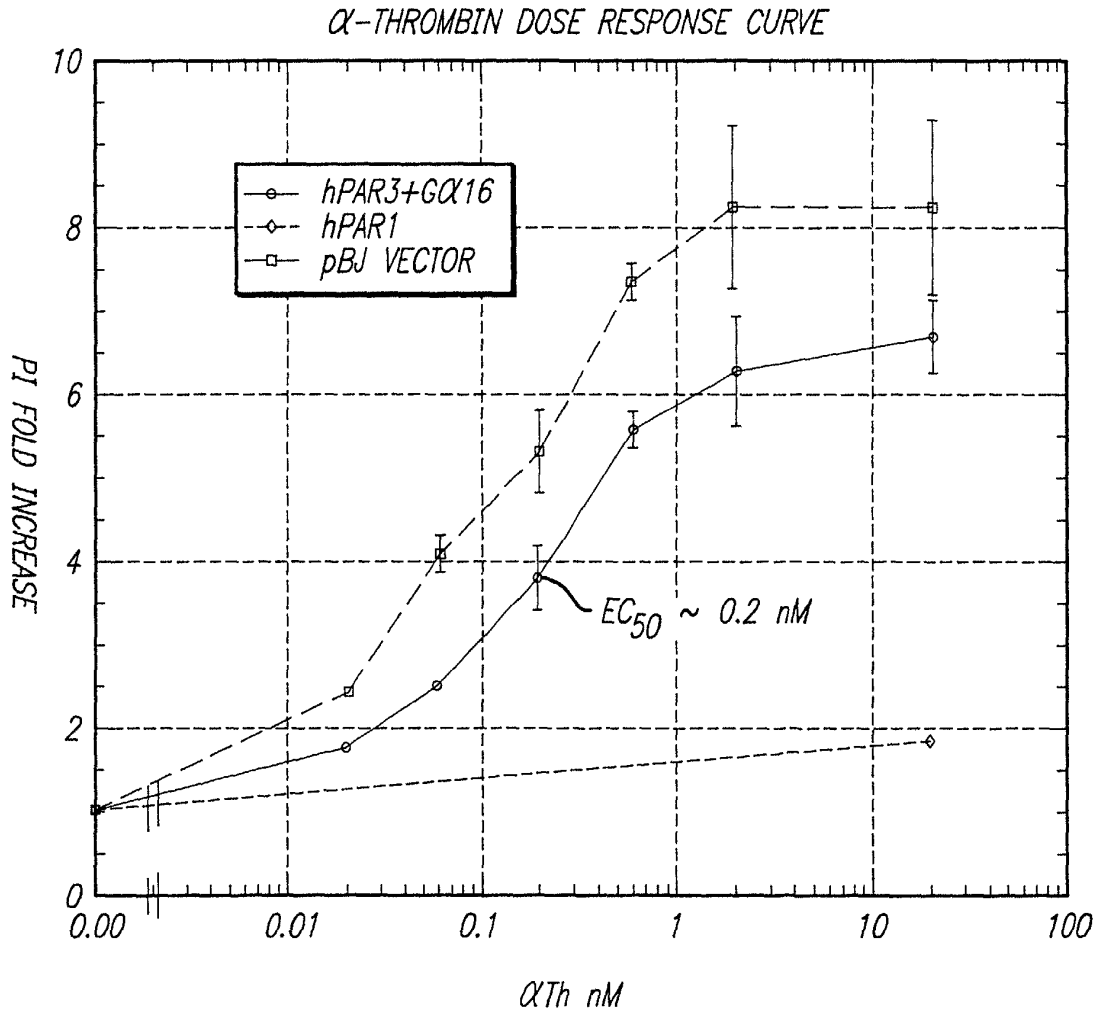
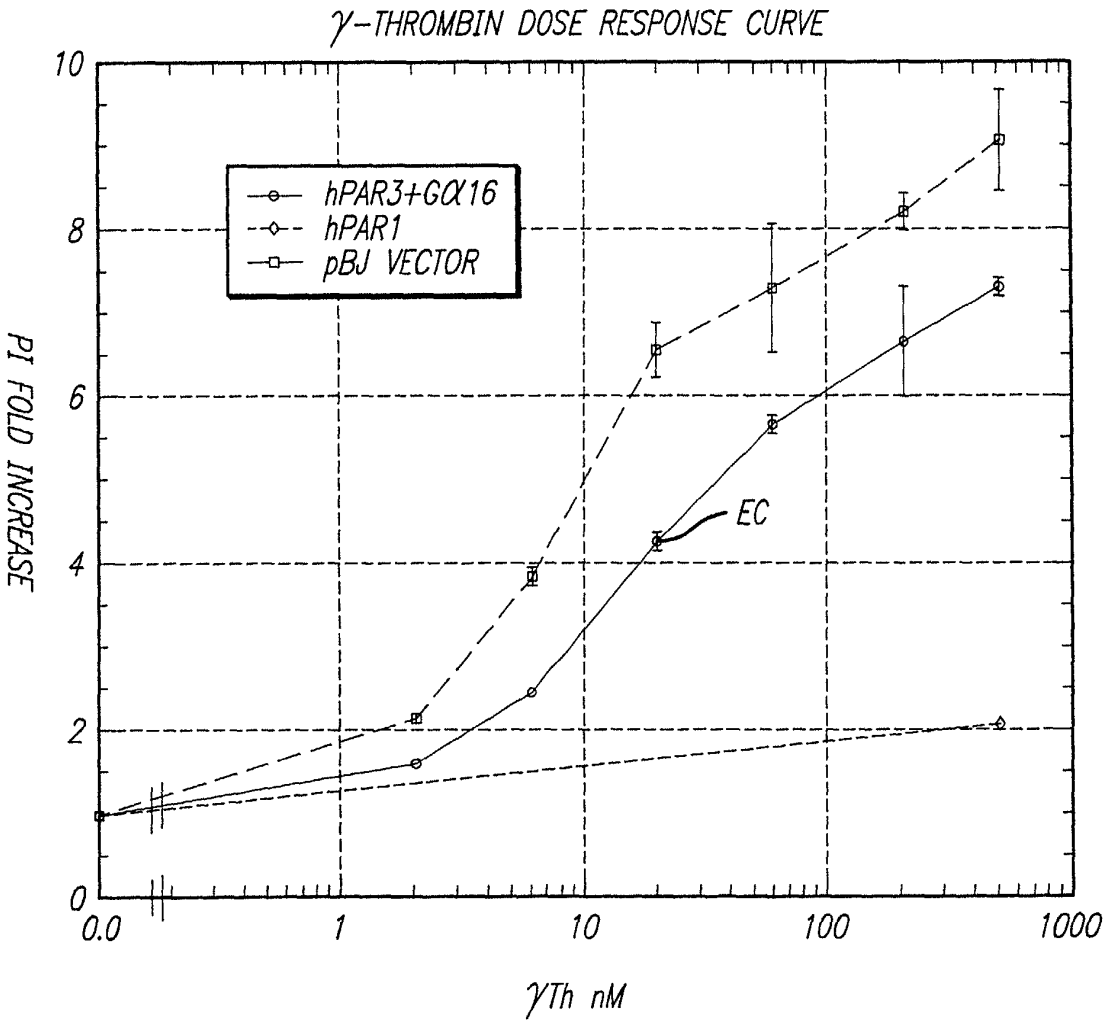

α-THROMBIN DOSE RESPONSE CURVE
hPAR3+Gα16
hPAR1
pBJ VECTOR
PI FOLD INCREASE
EC50 ~ 0.2 nM
αTh nM
0
2
4
6
8
10
0.00
0.01
0.1
1
10
100

FIG. 9


γ-THROMBIN DOSE RESPONSE CURVE
hPAR3+Gα16
hPAR1
pBJ VECTOR
EC
PI FOLD INCREASE
γTh nM
0
2
4
6
8
10
0.0
1
10
100
1000

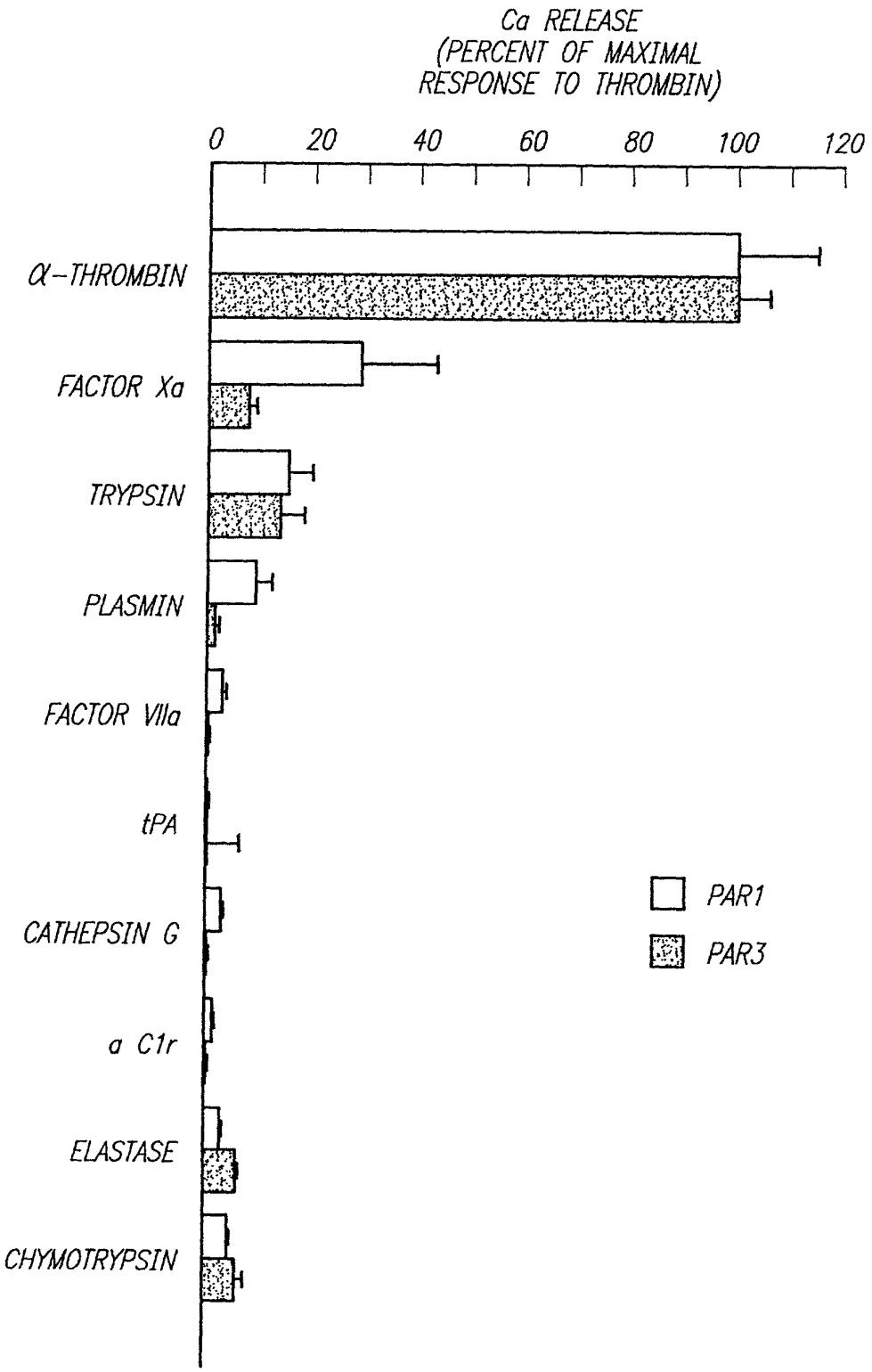

Ca RELEASE
(PERCENT OF MAXIMAL
RESPONSE TO THROMBIN)
0
20
40
60
80
100
120
α-THROMBIN
FACTOR Xa
TRYPSIN
PLASMIN
FACTOR VIIa
tPA
CATHEPSIN G
a C1r
ELASTASE
CHYMOTRYPSIN
PAR1
PAR3

FIG. 10

```
         10          20          30          40          50
TG ACT TTG TAT ACT TAA CAA CAT CCT GTA GCC GGG TCT CAG GAC ATC AAG
AC TGA AAC ATA TGA ATT GTT GTA GGA CAT CGG CCC AGA GTC CTG TAG TTC
    T   L   Y   T   *   Q   H   P   V   A   G   S   Q   D   I   K>

        60          70          80          90
ATG AAA ATC CTT ATC TTG GTT GCA GCT GGG CTG CTG TTT CTG CCA GTC
TAC TTT TAG GAA TAG AAC CAA CGT CGA CCC GAC GAC AAA GAC GGT CAG
 M   K   I   L   I   L   V   A   A   G   L   L   F   L   P   V>

100         110         120         130         140
ACT GTT TGC CAA AGT GGC ATA AAT GTT TCA GAC AAC TCA GCA AAG CCA
TGA CAA ACG GTT TCA CCG TAT TTA CAA AGT CTG TTG AGT CGT TTC GGT
 T   V   C   Q   S   G   I   N   V   S   D   N   S   A   K   P>

    150         160         170         180         190
ACC TTA ACT ATT AAG AGT TTT AAT GGG GGT CCC CAA AAT ACC TTT GAA
TGG AAT TGA TAA TTC TCA AAA TTA CCC CCA GGG GTT TTA TGG AAA CTT
 T   L   T   I   K   S   F   N   G   G   P   Q   N   T   F   E>

        200         210         220         230         240
GAA TTC CCA CTT TCT GAC ATA GAG GGC TGG ACA GGA GCC ACC ACA ACT
CTT AAG GGT GAA AGA CTG TAT CTC CCG ACC TGT CCT CGG TGG TGT TGA
 E   F   P   L   S   D   I   E   G   W   T   G   A   T   T   T>

            250         260         270         280         290
ATA AAA GCG GAG TGT CCC GAG GAC AGT ATT TCA ACT CTC CAC GTG AAT
TAT TTT CGC CTC ACA GGG CTC CTG TCA TAA AGT TGA GAG GTG CAC TTA
 I   K   A   E   C   P   E   D   S   I   S   T   L   H   V   N>

            300         310         320         330
AAT GCT ACC ATA GGA TAC CTG AGA AGT TCC TTA AGT ACC CAA GTG ATA
TTA CGA TGG TAT CCT ATG GAC TCT TCA AGG AAT TCA TGG GTT CAC TAT
 N   A   T   I   G   Y   L   R   S   S   L   S   T   Q   V   I>

340         350         360         370         380
CCT GCC ATC TAT ATC CTG CTG TTT GTG GTT GGT GTA CCA TCC AAC ATC
GGA CGG TAG ATA TAG GAC GAC AAA CAC CAA CCA CAT GGT AGG TTG TAG
 P   A   I   Y   I   L   L   F   V   V   G   V   P   S   N   I>

    390         400         410         420         430
GTG ACC CTG TGG AAA CTC TCC TTA AGG ACC AAA TCC ATC AGT CTG GTC
CAC TGG GAC ACC TTT GAG AGG AAT TCC TGG TTT AGG TAG TCA GAC CAG
 V   T   L   W   K   L   S   L   R   T   K   S   I   S   L   V>

        440         450         460         470         480
ATC TTT CAC ACC AAC CTG GCC ATC GCA GAT CTC CTT TTC TGT GTC ACA
TAG AAA GTG TGG TTG GAC CGG TAG CGT CTA GAG GAA AAG ACA CAG TGT
 I   F   H   T   N   L   A   I   A   D   L   L   F   C   V   T>
```

FIG. 1

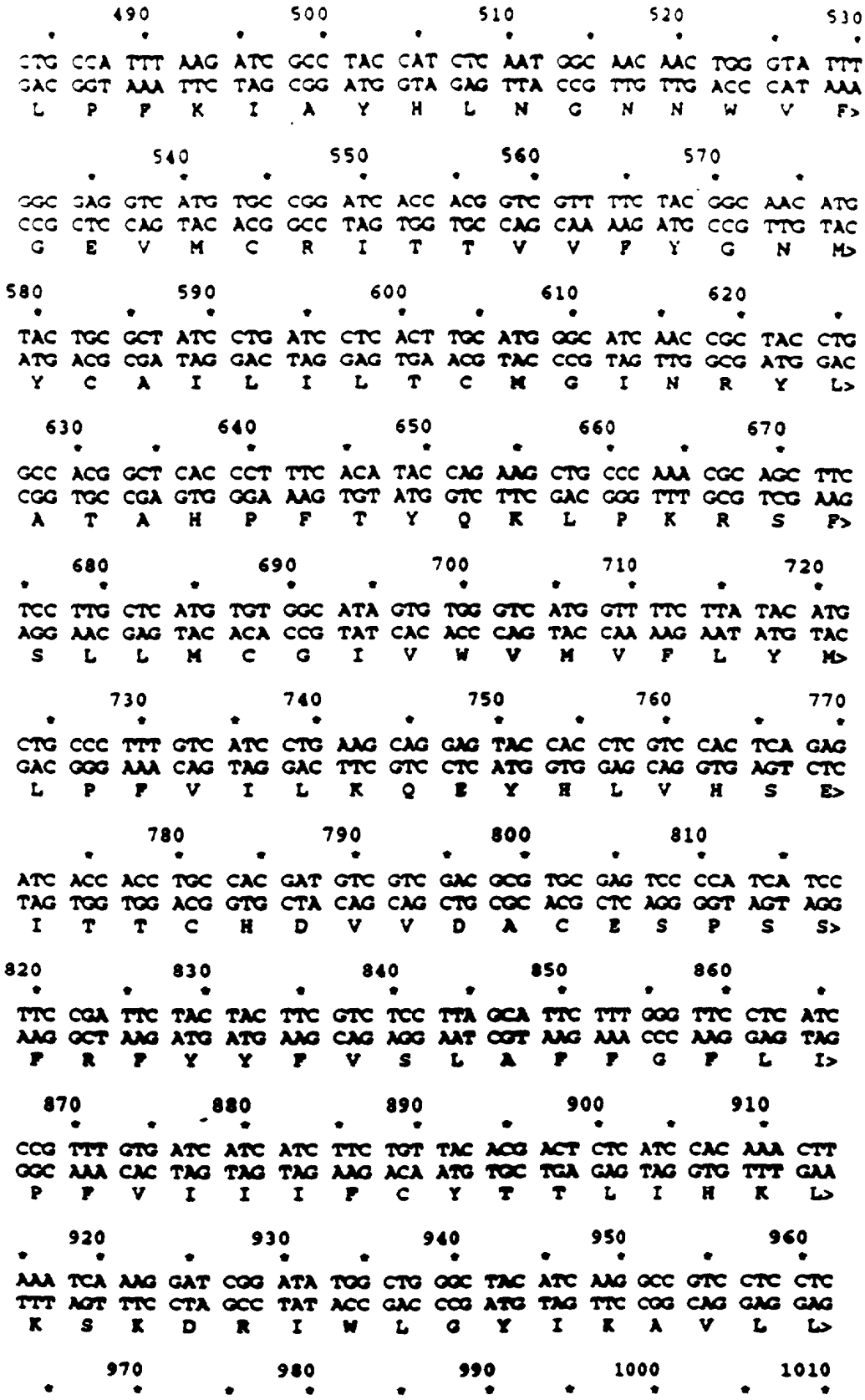

490 500 510 520 530
CTG CCA TTT AAG ATC GCC TAC CAT CTC AAT GGC AAC AAC TGG GTA TTT
GAC GGT AAA TTC TAG CGG ATG GTA GAG TTA CCG TTG TTG ACC CAT AAA
L P F K I A Y H L N G N N W V F>
540 550 560 570
GGC GAG GTC ATG TGC CGG ATC ACC ACG GTC GTT TTC TAC GGC AAC ATG
CCG CTC CAG TAC ACG GCC TAG TGG TGC CAG CAA AAG ATG CCG TTG TAC
G E V M C R I T T V V F Y G N M>
580 590 600 610 620
TAC TGC GCT ATC CTG ATC CTC ACT TGC ATG GGC ATC AAC CGC TAC CTG
ATG ACG CGA TAG GAC TAG GAG TGA ACG TAC CCG TAG TTG GCG ATG GAC
Y C A I L I L T C M G I N R Y L>
630 640 650 660 670
GCC ACG GCT CAC CCT TTC ACA TAC CAG AAG CTG CCC AAA CGC AGC TTC
CGG TGC CGA GTG GGA AAG TGT ATG GTC TTC GAC GGG TTT GCG TCG AAG
A T A H P F T Y Q K L P K R S F>
680 690 700 710 720
TCC TTG CTC ATG TGT GGC ATA GTG TGG GTC ATG GTT TTC TTA TAC ATG
AGG AAC GAG TAC ACA CCG TAT CAC ACC CAG TAC CAA AAG AAT ATG TAC
S L L M C G I V W V M V F L Y M>
730 740 750 760 770
CTG CCC TTT GTC ATC CTG AAG CAG GAG TAC CAC CTC GTC CAC TCA GAG
GAC GGG AAA CAG TAG GAC TTC GTC CTC ATG GTG GAG CAG GTG AGT CTC
L P F V I L K Q E Y H L V H S E>
780 790 800 810
ATC ACC ACC TGC CAC GAT GTC GTC GAC GCG TGC GAG TCC CCA TCA TCC
TAG TGG TGG ACG GTG CTA CAG CAG CTG CGC ACG CTC AGG GGT AGT AGG
I T T C H D V V D A C E S P S S>
820 830 840 850 860
TTC CGA TTC TAC TAC TTC GTC TCC TTA GCA TTC TTT GGG TTC CTC ATC
AAG GCT AAG ATG ATG AAG CAG AGG AAT CGT AAG AAA CCC AAG GAG TAG
F R F Y Y F V S L A F F G F L I>
870 880 890 900 910
CCG TTT GTG ATC ATC ATC TTC TGT TAC ACG ACT CTC ATC CAC AAA CTT
GGC AAA CAC TAG TAG TAG AAG ACA ATG TGC TGA GAG TAG GTG TTT GAA
P F V I I I F C Y T T L I H K L>
920 930 940 950 960
AAA TCA AAG GAT CGG ATA TGG CTG GGC TAC ATC AAG GCC GTC CTC CTC
TTT AGT TTC CTA GCC TAT ACC GAC CCG ATG TAG TTC CGG CAG GAG GAG
K S K D R I W L G Y I K A V L L>
970 980 990 1000 1010

FIG. 1 2 of 3

```
ATC CTT GTG ATT TTC ACA ATT TGC TTT GCC CCC ACC AAC ATC ATA CTC
TAG GAA CAC TAA AAG TGT TAA ACG AAA CGG GGG TGG TTG TAG TAT GAG
 I   L   V   I   F   T   I   C   F   A   P   T   N   I   I   L>

        1020        1030        1040        1050

GTA ATC CAC CAT GCC AAC TAC TAC TAC CAC AAT ACC GAC AGC TTG TAC
CAT TAG GTG GTA CGG TTG ATG ATG ATG GTG TTA TGG CTG TCG AAC ATG
 V   I   H   H   A   N   Y   Y   Y   H   N   T   D   S   L   Y>

1060        1070        1080        1090        1100

TTT ATG TAT CTT ATT GCT CTG TGC CTG GGG AGC CTG AAT AGC TGC CTA
AAA TAC ATA GAA TAA CGA GAC ACG GAC CCC TCG GAC TTA TCG ACG GAT
 F   M   Y   L   I   A   L   C   L   G   S   L   N   S   C   L>

  1110        1120        1130        1140        1150

GAT CCA TTC CTT TAC TTT GTC ATG TCG AAA GTT GTA GAT CAG CTT AAT
CTA GGT AAG GAA ATG AAA CAG TAC AGC TTT CAA CAT CTA GTC GAA TTA
 D   P   F   L   Y   F   V   M   S   K   V   V   D   Q   L   N>

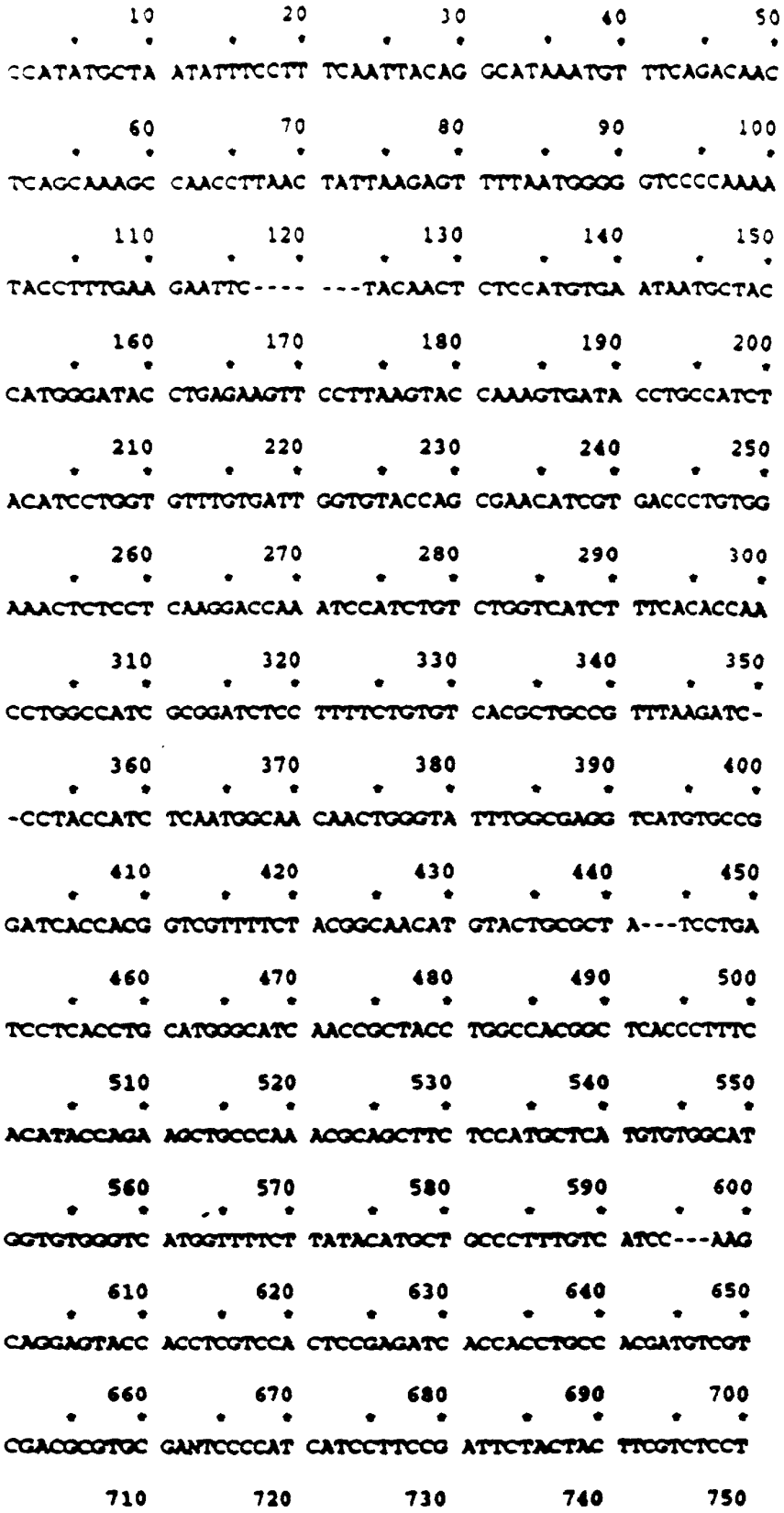
    1160        1170        1180        1190        1200

CCT TAG TCG GCA ATG GCA AGA CCA CTT TAG AGA CCA AGG AGA GAT ATC
GGA ATC AGC CGT TAC CGT TCT GGT GAA ATC TCT GGT TCC TCT CTA TAG
 P   *   S   A   M   A   R   P   L   *   R   P   R   R   D   I>

      1210        1220

TGG GAA GAC ATA CAT GCT TGG C
ACC CTT CTG TAT GTA CGA ACC G
 W   E   D   I   H   A   W   X>
```

FIG. 1 **3 of 3**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60 70 80 90 100
TCAGCAAAGC CAACCTTAAC TATTAAGAGT TTTAATGGGG GTCCCCAAAA
110 120 130 140 150
TACCTTTGAA GAATTC---- ---TACAACT CTCCATGTGA ATAATGCTAC
160 170 180 190 200
CATGGGATAC CTGAGAAGTT CCTTAAGTAC CAAAGTGATA CCTGCCATCT
210 220 230 240 250
ACATCCTGGT GTTTGTGATT GGTGTACCAG CGAACATCGT GACCCTGTGG
260 270 280 290 300
AAACTCTCCT CAAGGACCAA ATCCATCTGT CTGGTCATCT TTCACACCAA
310 320 330 340 350
CCTGGCCATC GCGGATCTCC TTTTCTGTGT CACGCTGCCG TTTAAGATC-
360 370 380 390 400
-CCTACCATC TCAATGGCAA CAACTGGGTA TTTGGCGAGG TCATGTGCCG
410 420 430 440 450
GATCACCACG GTCGTTTTCT ACGGCAACAT GTACTGCGCT A---TCCTGA
460 470 480 490 500
TCCTCACCTG CATGGGCATC AACCGCTACC TGGCCACGGC TCACCCTTTC
510 520 530 540 550
ACATACCAGA AGCTGCCCAA ACGCAGCTTC TCCATGCTCA TGTGTGGCAT
560 570 580 590 600
GGTGTGGGTC ATGGTTTTCT TATACATGCT GCCCTTTGTC ATCC---AAG
610 620 630 640 650
CAGGAGTACC ACCTCGTCCA CTCCGAGATC ACCACCTGCC ACGATGTCGT
660 670 680 690 700
CGACGCGTGC GANTCCCCAT CATCCTTCCG ATTCTACTAC TTCGTCTCCT
710 720 730 740 750

FIG. 2 1 of 2

```
TAGCATTCTT TGGGTTCCTC ATCCCGTTTG TGATCATCAT CTTCTGTTAC

ACGACTCTCA TCCACAAACT TAAATCAAAA GATCNGATAT GGCTGGGCTA

CATCAAGGCC GTCCTCCTCA TCCTTGTGAA TTTCACCATC TGCTTCCCCC

CCACCAAG-- ----GATATC TGGGAAGACG TACATGCTTG GCTGACTTGT

GCATGGCACC ATCAGCTCAA TTTTTAATTT TTTAATTTTA ATTTAATTTA

ATTTTATGTT TTTGAGACAG AGCCTCACTG TGTAGTCCTG GCTGGCCTGG

CTGGTTCTCT ATTTAGACCA GGTTAGCCTT GAACTCACAG AGATCTGCCT

GCTTCTGCCT CCCAAGTGCT GGGTTCAACC AGGTCTGGCA AGCGCTCCAT

TTTTCAGCTC CTCTGCAACA GTGC
```

FIG. 2 2 of 2

```
        10          20          30          40
TGC TCC ATG ATT TTA CAG ATT TCA TAA CGT TTA AGA GAC GGG ACT CAG
ACG AGG TAC TAA AAT GTC TAA AGT ATT GCA AAT TCT CTG CCC TGA GTC
 C   S   M   I   L   Q   I   S   *   R   L   R   D   G   T   Q>

50          60          70          80          90
GTC ATC AAA ATG AAA GCC CTC ATC TTT GCA GCT GCT GGC CTC CTG CTT
CAG TAG TTT TAC TTT CGG GAG TAG AAA CGT CGA CGA CCG GAG GAC GAA
 V   I   K   M   K   A   L   I   F   A   A   A   G   L   L   L>

  100         110         120         130         140
CTG TTG CCC ACT TTT TGT CAG AGT GGC ATG GAA AAT GAT ACA AAC AAC
GAC AAC GGG TGA AAA ACA GTC TCA CCG TAC CTT TTA CTA TGT TTG TTG
 L   L   P   T   F   C   Q   S   G   M   E   N   D   T   N   N>

    150         160         170         180         190
TTG GCA AAG CCA ACC TTA CCC ATT AAG ACC TTT CGT GGA GCT CCC CCA
AAC CGT TTC GGT TGG AAT GGG TAA TTC TGG AAA GCA CCT CGA GGG GGT
 L   A   K   P   T   L   P   I   K   T   F   R   G   A   P   P>

      200         210         220         230         240
AAT TCT TTT GAA GAG TTC CCC TTT TCT GCC TTG GAA GGC TGG ACA GGA
TTA AGA AAA CTT CTC AAG GGG AAA AGA CGG AAC CTT CCG ACC TGT CCT
 N   S   F   E   E   F   P   F   S   A   L   E   G   W   T   G>

        250         260         270         280
GCC ACG ATT ACT GTA AAA ATT AAG TGC CCT GAA GAA AGT GCT TCA CAT
CGG TGC TAA TGA CAT TTT TAA TTC ACG GGA CTT CTT TCA CGA AGT GTA
 A   T   I   T   V   K   I   K   C   P   E   E   S   A   S   H>

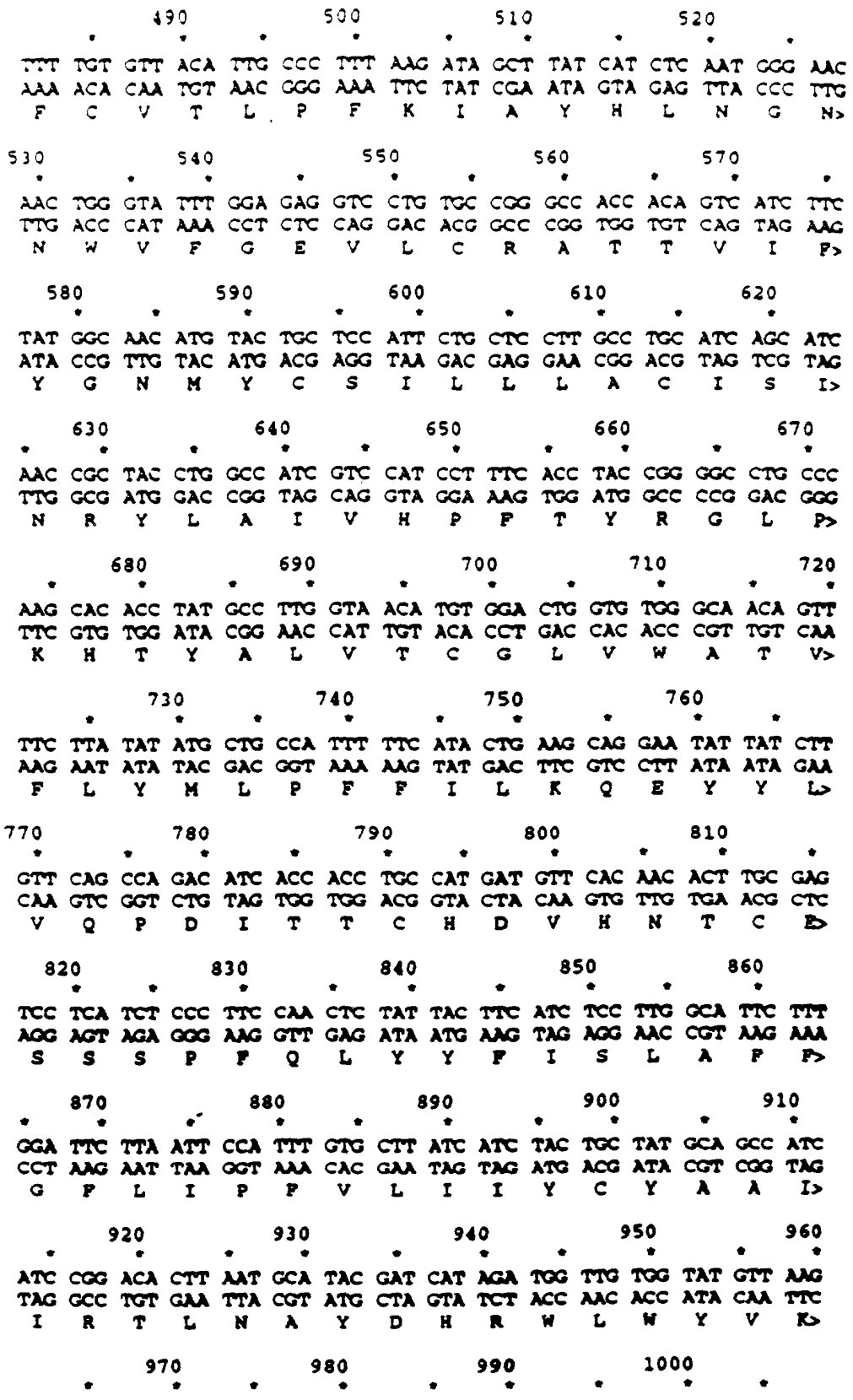
290         300         310         320         330
CTC CAT GTG AAA AAT GCT ACC ATG GGG TAC CTG ACC AGC TCC TTA AGT
GAG GTA CAC TTT TTA CGA TGG TAC CCC ATG GAC TGG TCG AGG AAT TCA
 L   H   V   K   N   A   T   M   G   Y   L   T   S   S   L   S>

  340         350         360         370         380
ACT AAA CTG ATA CCT GCC ATC TAC CTC CTG GTG TTT GTA GTT GGT GTC
TGA TTT GAC TAT GGA CGG TAG ATG GAG GAC CAC AAA CAT CAA CCA CAG
 T   K   L   I   P   A   I   Y   L   L   V   F   V   V   G   V>

    390         400         410         420         430
CCG GCC AAT GCT GTG ACC CTG TGG ATG CTT TTC TTC AGG ACC AGA TCC
GGC CGG TTA CGA CAC TGG GAC ACC TAC GAA AAG AAG TCC TGG TCT AGG
 P   A   N   A   V   T   L   W   M   L   F   F   R   T   R   S>

        440         450         460         470         480
ATC TGT ACC ACT GTA TTC TAC ACC AAC CTG GCC ATT GCA GAT TTT CTT
TAG ACA TGG TGA CAT AAG ATG TGG TTG GAC CGG TAA CGT CTA AAA GAA
 I   C   T   T   V   F   Y   T   N   L   A   I   A   D   F   L>
```

FIG. 3 1 of 3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AAA ACA CAA TGT AAC GGG AAA TTC TAT CGA ATA GTA GAG TTA CCC TTG
F C V T L P F K I A Y H L N G N>
530 540 550 560 570
AAC TGG GTA TTT GGA GAG GTC CTG TGC CGG GCC ACC ACA GTC ATC TTC
TTG ACC CAT AAA CCT CTC CAG GAC ACG GCC CGG TGG TGT CAG TAG AAG
N W V F G E V L C R A T T V I F>
580 590 600 610 620
TAT GGC AAC ATG TAC TGC TCC ATT CTG CTC CTT GCC TGC ATC AGC ATC
ATA CCG TTG TAC ATG ACG AGG TAA GAC GAG GAA CGG ACG TAG TCG TAG
Y G N M Y C S I L L L A C I S I>
630 640 650 660 670
AAC CGC TAC CTG GCC ATC GTC CAT CCT TTC ACC TAC CGG GGC CTG CCC
TTG GCG ATG GAC CGG TAG CAG GTA GGA AAG TGG ATG GCC CCG GAC GGG
N R Y L A I V H P F T Y R G L P>
680 690 700 710 720
AAG CAC ACC TAT GCC TTG GTA ACA TGT GGA CTG GTG TGG GCA ACA GTT
TTC GTG TGG ATA CGG AAC CAT TGT ACA CCT GAC CAC ACC CGT TGT CAA
K H T Y A L V T C G L V W A T V>
730 740 750 760
TTC TTA TAT ATG CTG CCA TTT TTC ATA CTG AAG CAG GAA TAT TAT CTT
AAG AAT ATA TAC GAC GGT AAA AAG TAT GAC TTC GTC CTT ATA ATA GAA
F L Y M L P F F I L K Q E Y Y L>
770 780 790 800 810
GTT CAG CCA GAC ATC ACC ACC TGC CAT GAT GTT CAC AAC ACT TGC GAG
CAA GTC GGT CTG TAG TGG TGG ACG GTA CTA CAA GTG TTG TGA ACG CTC
V Q P D I T T C H D V H N T C E>
820 830 840 850 860
TCC TCA TCT CCC TTC CAA CTC TAT TAC TTC ATC TCC TTG GCA TTC TTT
AGG AGT AGA GGG AAG GTT GAG ATA ATG AAG TAG AGG AAC CGT AAG AAA
S S S P F Q L Y Y F I S L A F F>
870 880 890 900 910
GGA TTC TTA ATT CCA TTT GTG CTT ATC ATC TAC TGC TAT GCA GCC ATC
CCT AAG AAT TAA GGT AAA CAC GAA TAG TAG ATG ACG ATA CGT CGG TAG
G F L I P F V L I I Y C Y A A I>
920 930 940 950 960
ATC CGG ACA CTT AAT GCA TAC GAT CAT AGA TGG TTG TGG TAT GTT AAG
TAG GCC TGT GAA TTA CGT ATG CTA GTA TCT ACC AAC ACC ATA CAA TTC
I R T L N A Y D H R W L W Y V K>
970 980 990 1000

FIG. 3 2 of 3

```
GCG AGT CTC CTC ATC CTT GTG ATT TTT ACC ATT TGC TTT GCT CCA AGC
CGC TCA GAG GAG TAG GAA CAC TAA AAA TGG TAA ACG AAA CGA GGT TCG
 A   S   L   L   I   L   V   I   F   T   I   C   F   A   P   S>

1010        1020        1030        1040        1050
AAT ATT ATT CTT ATT ATT CAC CAT GCT AAC TAC TAC TAC AAC AAC ACT
TTA TAA TAA GAA TAA TAA GTG GTA CGA TTG ATG ATG ATG TTG TTG TGA
 N   I   I   L   I   I   H   H   A   N   Y   Y   Y   N   N   T>

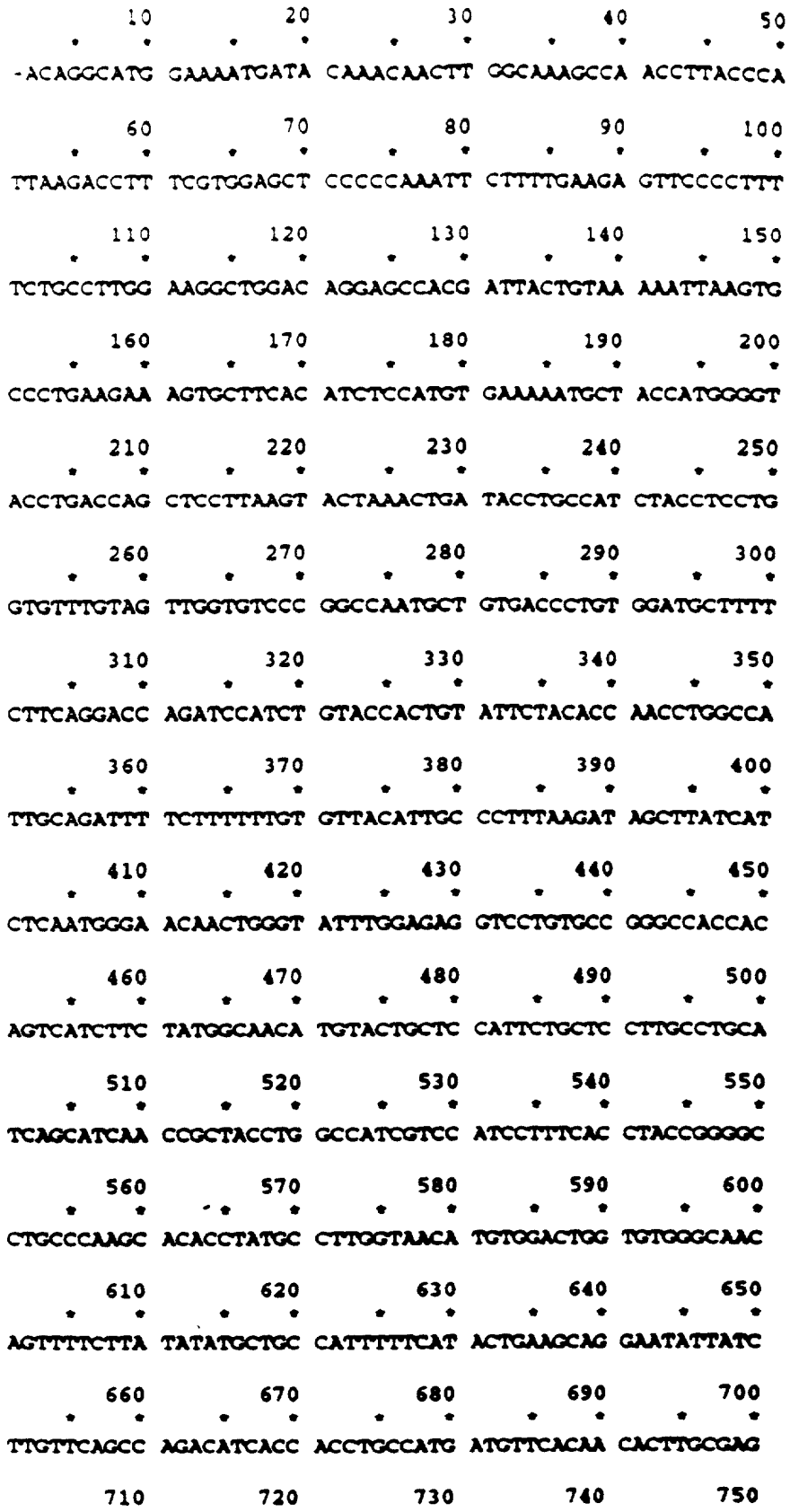
1060        1070        1080        1090        1100
GAT GGC TTA TAT TTT ATA TAT CTC ATA GCT TTG TGC CTG GGT AGT CTT
CTA CCG AAT ATA AAA TAT ATA GAG TAT CGA AAC ACG GAC CCA TCA GAA
 D   G   L   Y   F   I   Y   L   I   A   L   C   L   G   S   L>

1110        1120        1130        1140        1150
AAT AGT TGC TTA GAT CCA TTC CTT TAT TTT CTC ATG TCA AAA ACC AGA
TTA TCA ACG AAT CTA GGT AAG GAA ATA AAA GAG TAC AGT TTT TGG TCT
 N   S   C   L   D   P   F   L   Y   F   L   M   S   K   T   R>

1160        1170        1180        1190        1200
AAT CAC TCC ACT GCT TAC CTT ACA AAA TAG TGA AAT GAT CTT AGA GAA
TTA GTG AGG TGA CGA ATG GAA TGT TTT ATC ACT TTA CTA GAA TCT CTT
 N   H   S   T   A   Y   L   T   K   *   *   N   D   L   R   E>

1210        1220
CAA GGA CAG CCA TCA CAG AGA ACG
GTT CCT GTC GGT AGT GTC TCT TGC
 Q   G   Q   P   S   Q   R   T>
```

FIG. 3 **3 of 3**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60 70 80 90 100
TTAAGACCTT TCGTGGAGCT CCCCCAAATT CTTTTGAAGA GTTCCCCTTT
110 120 130 140 150
TCTGCCTTGG AAGGCTGGAC AGGAGCCACG ATTACTGTAA AAATTAAGTG
160 170 180 190 200
CCCTGAAGAA AGTGCTTCAC ATCTCCATGT GAAAAATGCT ACCATGGGGT
210 220 230 240 250
ACCTGACCAG CTCCTTAAGT ACTAAACTGA TACCTGCCAT CTACCTCCTG
260 270 280 290 300
GTGTTTGTAG TTGGTGTCCC GGCCAATGCT GTGACCCTGT GGATGCTTTT
310 320 330 340 350
CTTCAGGACC AGATCCATCT GTACCACTGT ATTCTACACC AACCTGGCCA
360 370 380 390 400
TTGCAGATTT TCTTTTTTGT GTTACATTGC CCTTTAAGAT AGCTTATCAT
410 420 430 440 450
CTCAATGGGA ACAACTGGGT ATTTGGAGAG GTCCTGTGCC GGGCCACCAC
460 470 480 490 500
AGTCATCTTC TATGGCAACA TGTACTGCTC CATTCTGCTC CTTGCCTGCA
510 520 530 540 550
TCAGCATCAA CCGCTACCTG GCCATCGTCC ATCCTTTCAC CTACCGGGGC
560 570 580 590 600
CTGCCCAAGC ACACCTATGC CTTGGTAACA TGTGGACTGG TGTGGGCAAC
610 620 630 640 650
AGTTTTCTTA TATATGCTGC CATTTTTCAT ACTGAAGCAG GAATATTATC
660 670 680 690 700
TTGTTCAGCC AGACATCACC ACCTGCCATG ATGTTCACAA CACTTGCGAG
710 720 730 740 750

FIG. 4 1 of 2

```
TCCTCATCTC CCTTCCAACT CTATTACTTC ATCTCCTTGG CATTCTTTGG

       760        770        780        790        800
ATTCTTAATT CCATTTGTGC TTATCATCTA CTGCTATGCA GCCATCATCC

       810        820        830        840        850
GGACACTTAA TGCATACGAT CATAGATGGT TGTGGTATGT TAAGGCGAGT

       860        870        880        890        900
CTCCTCATCC TTGTGATTTT TACCATTTGC TTTGCTCCAA GCAATATTAT

       910        920        930        940        950
TCTTATTATT CACCATGCTA ACTACTACTA CAACAACACT GATGGCTTAT

       960        970        980        990       1000
ATTTTATATA TCTCATAGCT TTGTGCCTGG GTAGTCTTAA TAGTTGCTTA

      1010       1020       1030       1040       1050
GATCCATTCC TTTATTTTCT CATGTCAAAA ACCAGAAATC ACTCCACTGC

      1060       1070       1080       1090       1100
TTACCTTACA AAATAGTGAA ATGATCTTAG AGAACAAGGA CAGCCATCAC

AGA
```

FIG. 4   2 of 2

FIG. 1

a. AMINO ACID SEQUENCE OF PROTEASE-ACTIVATED RECEPTOR 3, COMPARISON WITH PAR1 AND PAR2.

```
hPAR3-  1  MKA   LIFAAAGLLLLLP TFTQSGKENDTNNLAKP TLPIK/TFRGAPPN SFEEFPFSALEGWTGATITVKIKC     PEESASHLHVKNATMG
hPAR1-  1  MGPRR LLLVAACFSLCGP LLSARTRARRPESKATNATLDPR SFLLRNPNDKYEPFWEDEEKNESGLTEYRLVSINKSSPLQKQLPAFISEDASG
hPAR2-  1  MRSPSAAWLLGAAILLA ASLSCSGTIQG      TNRSSKGR SLIGKVDGTSHVTGKGVTV                                        ETVFSVDEFSAS

                    |----------TM1-----------|             |-----------TM2---------|                    |----------TM3-----------|
hPAR3-  87 YLTSSLSTKLIPAIYLLVFVVGVPANAVTLWMLFFRTR SICTTVFYTNLAIADFLFCVTLPFKIAYHLNGNNWVFGEVLCRATTVIFYGNMYCSILLLACISINRYLAI
hPAR1-  95 YLTSSWLTLFVPSVYTGVFVVSLPLNIMAIVVFILKMKVKKPAVVYMLHLATADVLFVSVLPFKISYYFSGSDWQFGSELCRFVTAAFYCNMYASILLMTVISIDRFLAV
hPAR2-  68 VLTGKLTTVFLPIVYTIVFVVGLPSNGMALWVFLFRTKKKHPAVIYMANLALADLLSVIWFPLKIAYHIHGNNWIYGEALCNVLIGFFYGNMYCSILFMTCLSVQRYWVI

                          |----------TM4----------|                                  |----------TM5-----------|
hPAR3- 196 VHPFTYRGLPKHTYALVTCGLVWATVFLYMLPFFILKQEYYLVQPDITTCHDVHNTCESSSPFQLYYFISLAFFGFLIPFVLIIYCYAAIIRTLNA        YDHRWLWYV
hPAR1- 205 VYPMQSLSWRTLGRASFTCLAIWALAIAGVVPLVLKEQTIQVPGLNITTCHDVLNETLLEG YYAYYFSAFSAVFFFVPLIISTVCYVSIIRCLSSSAVANRSKK  SRAL
hPAR2- 178 VNPMGHSRKKANIAIGI SLAIWLLILLVTIPLYVVKQTIFIPALNITTCHDVLPEQLLVGD MFNYFLSLAIGVFLFPAFLTASAYVLMIRMLRSSAMDENSEKKRKRAI

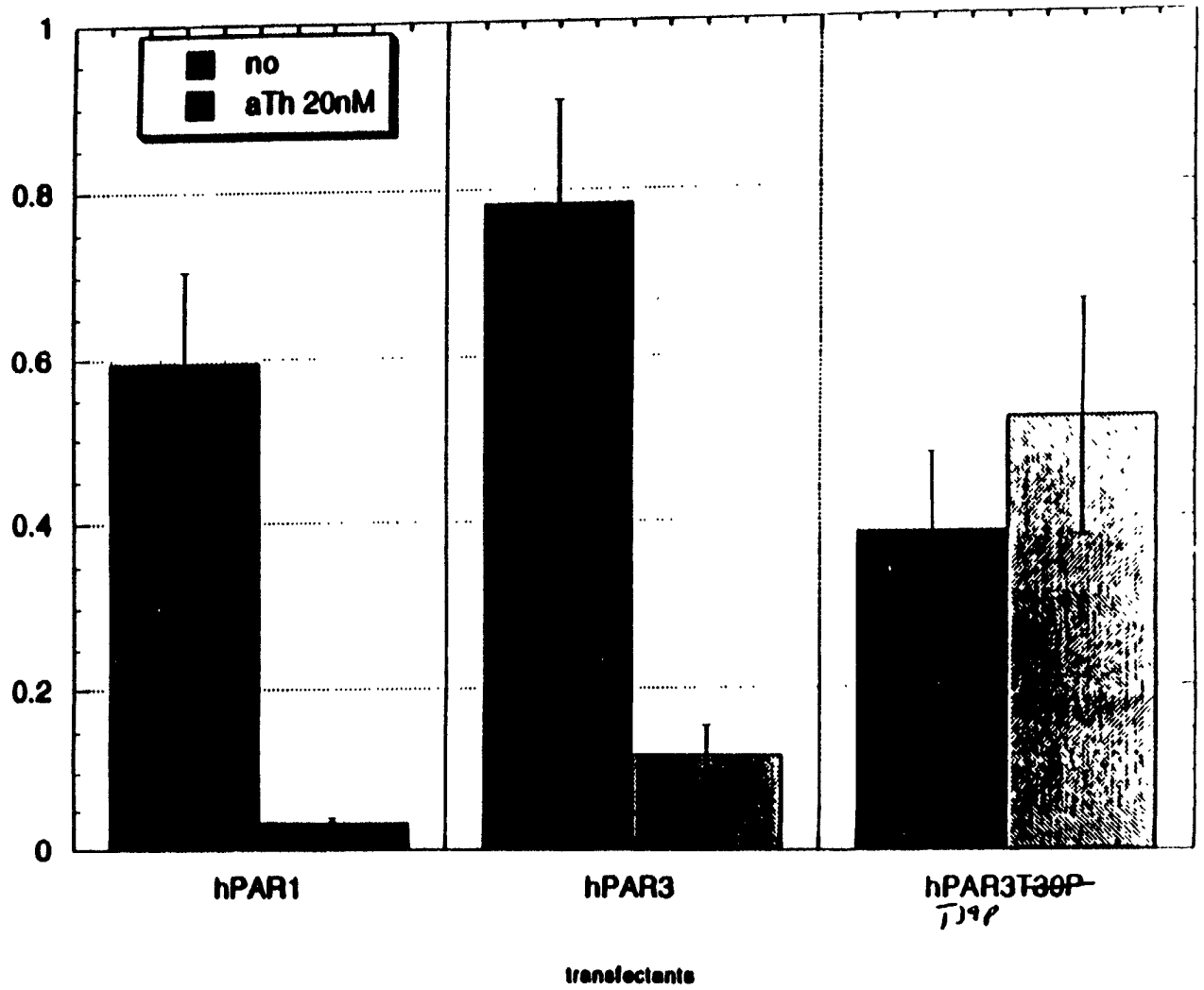
                |----------TM6--------|                |--------------TM7---------|
hPAR3- 301 KASLLILVIFTICFAPSNIILIIHHANYYYNNT DGLYFIYLIALCLGSLNSCLDPFLYFLMSKTRNHSTAYLTK
hPAR1- 313 FLSAAVFCIFIICFGPTNVLLIAHYSFLSHTSTTEAAYFAYLLCVCVSSISSCIDPLIYYYASSECQRYVYSILCCKESSDPSSYNSSGQLMASKMDTCSSNLNNSIYKKLLT
hPAR2- 287 KLIVTVLAMYLICFTPSNLLLVVHY FLIKSQGQSHVYALYIVALCLSTLNSCIDPFVYYFVSHDFRDHAKNALLCRSVRTVKQMQVSLTSKKHSRKSSSYSSSSTTVKTSY
```

b. FEATURES OF PAR AMINO TERMINAL EXODOMAINS.

```
Hirudin C-tail                    ..DFEEIPEEYLQ
hPAR3-  34-62  ..TLPIK  /  TFRGAPPN SFEEFPFSALEGWTGA..
hPAR1-  37-65  ..TLDPR  /  SFLLRNPNDKYEPFWEDEEKNESG..
hPAR2-  32-62  ..SSKGR  /  SLIGKVDGTSHVTGKGVTVETVFSVD..
                         ^
                   Cleavage site
```

FIGS. 5A & 5B

FIG. 6



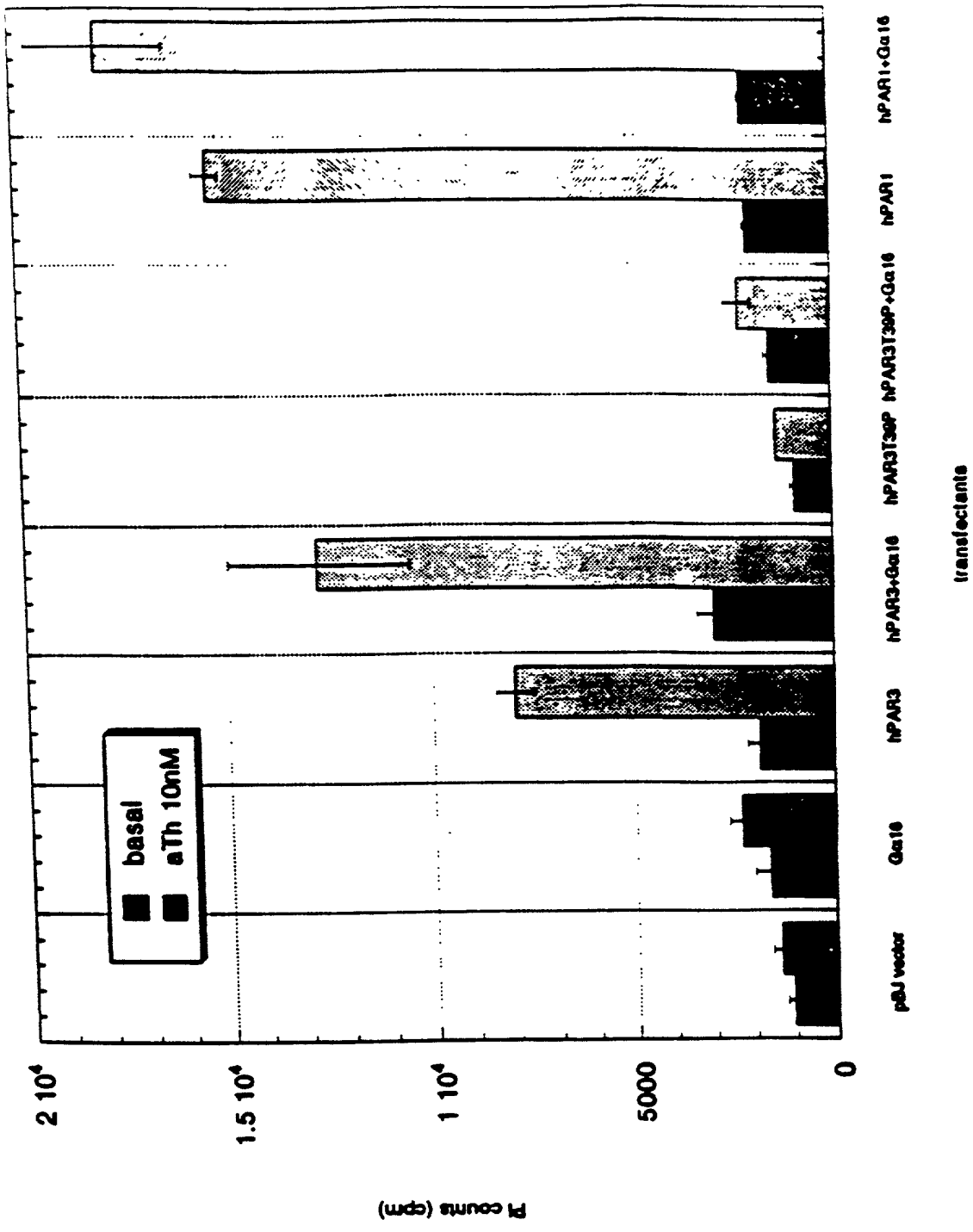

Signaling of PARs
effects of Gα16
basal
aTh 10nM
2 10⁴
1.5 10⁴
1 10⁴
5000
0
PI counts (cpm)
pBJ vector
Gα16
hPAR3
hPAR3+Gα16
hPAR3T30P
hPAR3T30P+Gα16
hPAR1
hPAR1+Gα16
transfectants

FIG. 7

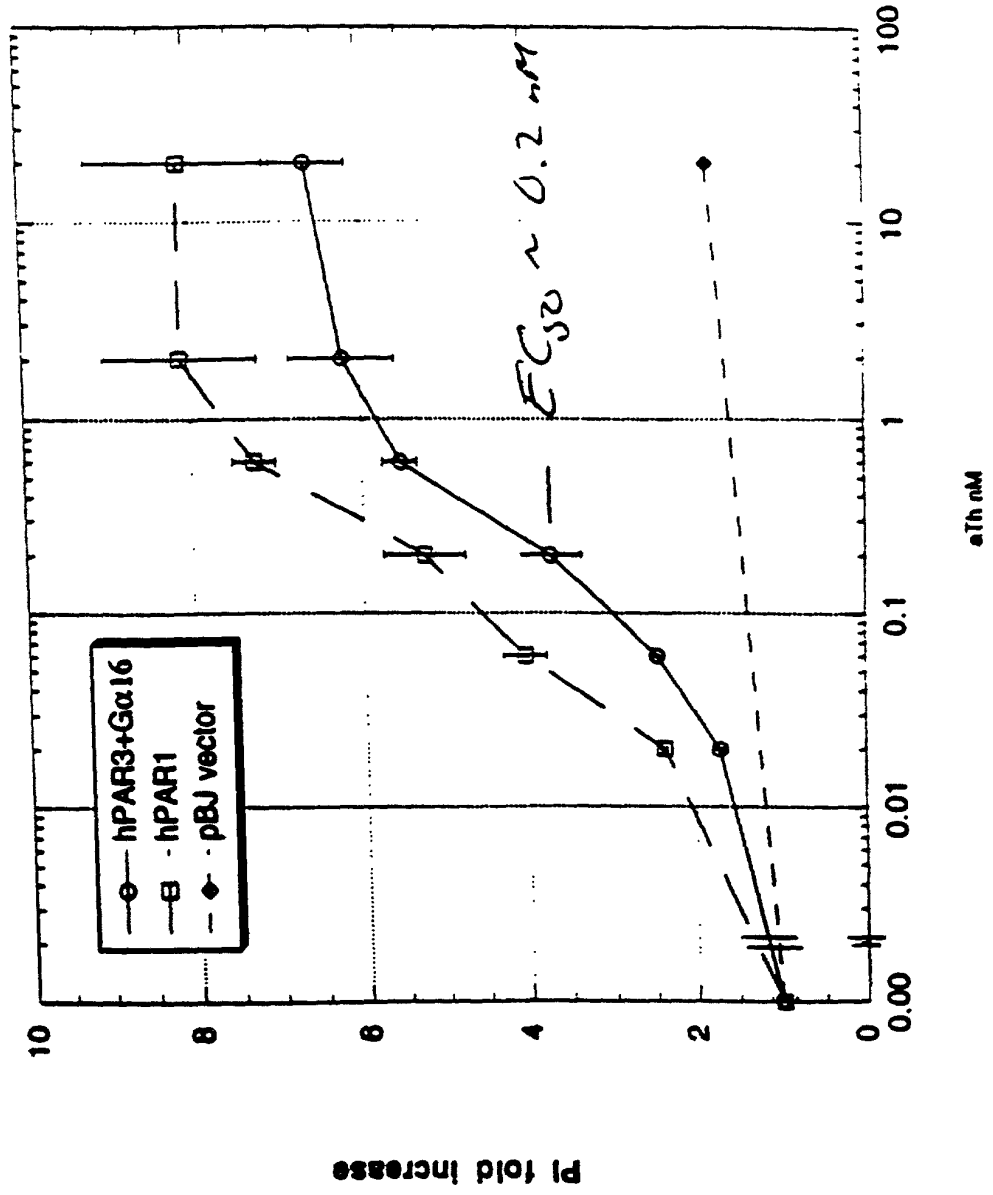

α-thrombin dose response curve
hPAR3+Gα16
hPAR1
pBJ vector
$EC_{50} \sim 0.2$ nM
PI fold increase
αTh nM
0
2
4
6
8
10
0.00
0.01
0.1
1
10
100

FIG. 8

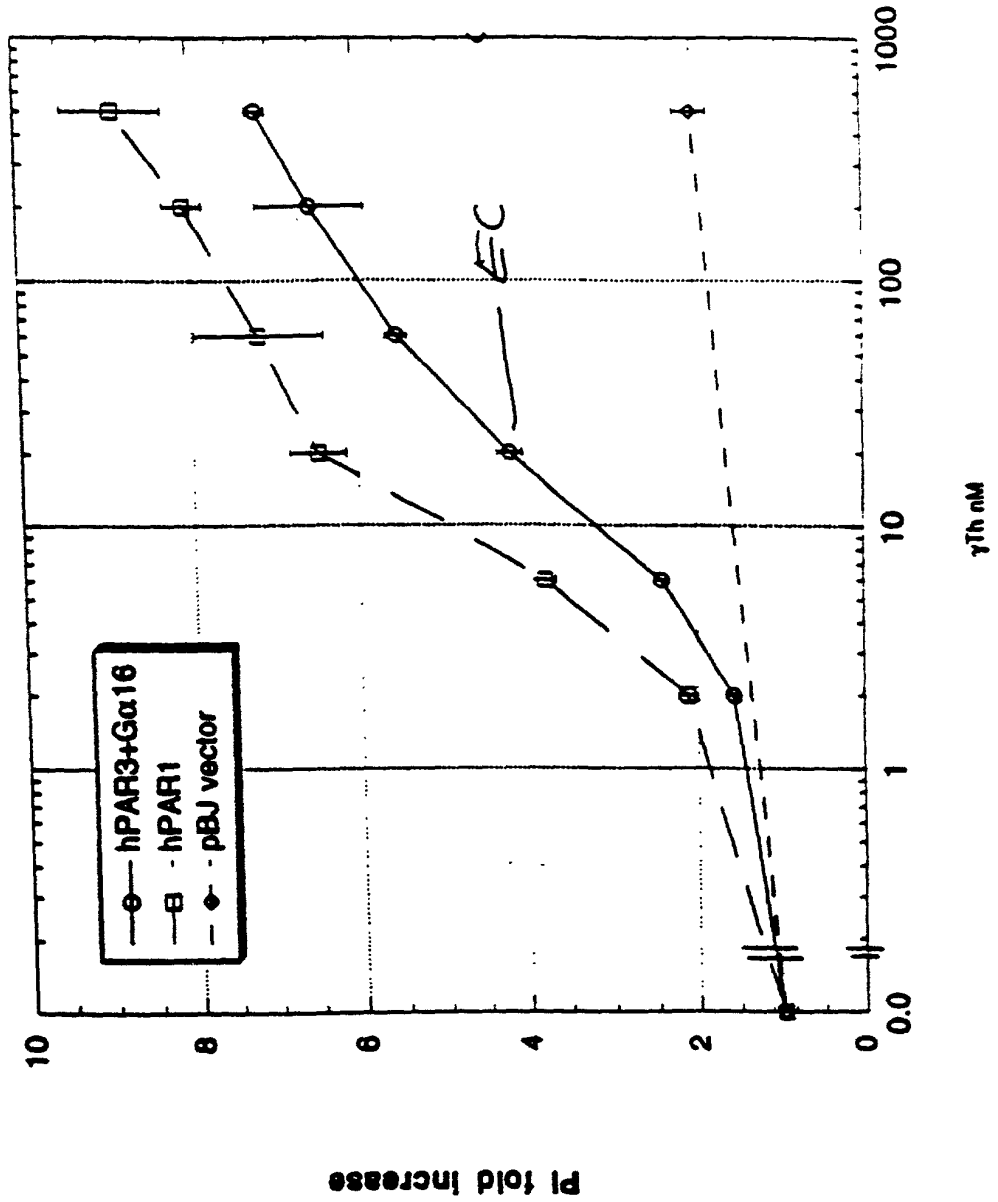

γ-thrombin dose response curve
hPAR3+Gα16
hPAR1
pBJ vector
EC
PI fold increase
γTh nM
0
2
4
6
8
10
0.0
1
10
100
1000

FIG. 9

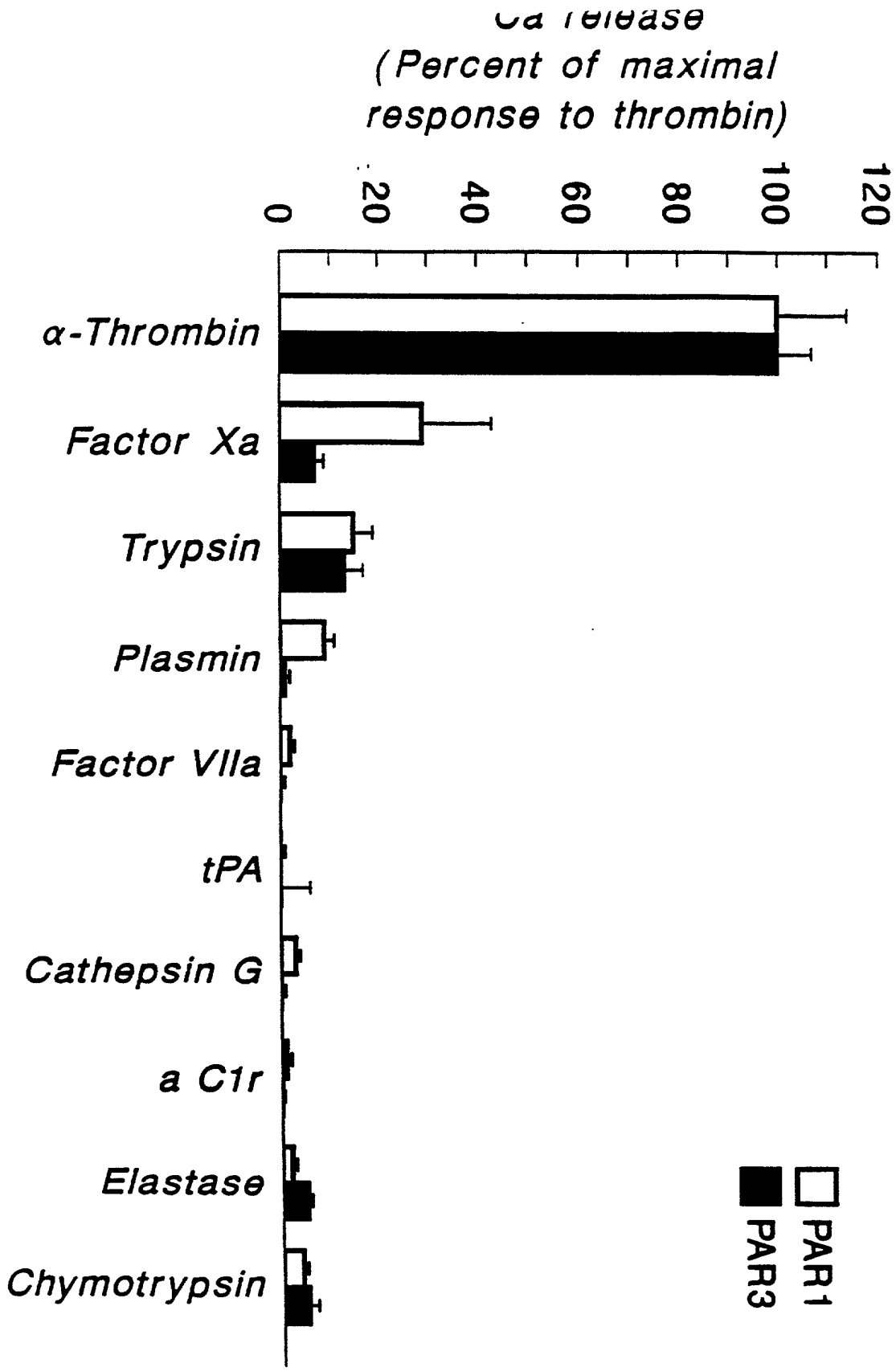

Ca release
(Percent of maximal
response to thrombin)
0
20
40
60
80
100
120
α-Thrombin
Factor Xa
Trypsin
Plasmin
Factor VIIa
tPA
Cathepsin G
a C1r
Elastase
Chymotrypsin
PAR1
PAR3

FIG. 10

PATENT

ATTORNEY DOCKET NO: 06510/060001

U.C. Case No. 97-045-1

COMBINED DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

PROTEASE ACTIVATED RECEPTOR 3 AND USES THEREOF

the specification of which

☐ is attached hereto.

X was filed on October 30, 1996

X as Application Serial No. 08/742,440

X and was amended on _____.

☐ was described and claimed in PCT International Application No. _____ filed on _____ and as amended under PCT Article 19 on _____.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose all information I know to be material to patentability in accordance with Title 37, Code of Federal Regulations, §1.56(a).

I hereby appoint the following attorneys and/or agents to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith: Karl Bozicevic, Reg. No. 28,807 Carol L. Francis, Reg. No. 36,513; Bret E. Field, Reg. No. 37,620; Pamela J. Sherwood, Reg. No. 36,677; Reginald J. Suyat, Reg. No. 28,172; Hans R. Troesch, Reg. No. 36,950; Grant D. Green, Reg. No. 31,259.

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

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